

MOLLUSCICIDAL AND MOSQUITOCIDAL ACTIVITIES OF THE ESSENTIAL OILS OF *Thymus capitatus* HOFF. ET LINK. AND *Marrubium vulgare* L.

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SUMMARY

Steam distillation of essential oils of aerial parts of *Thymus capitatus* and *Marrubium vulgare* L. collected at North coast of Egypt yielded 0.5% and 0.2%, respectively. Results of Gas chromatography-mass spectrometry analyses of the two samples identified 96.27% and 90.19% of the total oil composition for *T. capitatus* and *M. vulgare*, respectively. The two oil samples appeared dominated by the oxygenated constituents (88.22% for *T. capitatus* and 57.50% for *M. vulgare*), composed of phenols, mainly carvacrol (32.98%) and thymol (32.82%) in essential oil of *T. capitatus*, and thymol (34.55%) in essential oil of *M. vulgare*. It was evaluated the molluscicidal activity of *T. capitatus* and *M. vulgare* essential oils on adult and eggs of *Biomphalaria alexandrina* as well as their mosquitocidal activity on *Culex pipiens*. The LC₅₀ and LC₉₀ of *T. capitatus* essential oil against adult snails was 200 and 400 ppm/3hrs, respectively, while for *M. vulgare* it was 50 and 100 ppm/3hrs, respectively. Moreover, *M. vulgare* showed LC₁₀₀ ovicidal activity at 200 ppm/24 hrs while *T. capitatus* oil showed no ovicidal activity. It was verified mosquitocidal activity, with LC₅₀ and LC₉₀ of 100 and 200 ppm/12hrs respectively for larvae, and 200 and 400 ppm/12hrs respectively for pupae of *C. pipiens*.

KEYWORDS: Vector control; Plant products; *Thymus capitatus*; *Marrubium vulgare*.

INTRODUCTION

Vector borne diseases are major sources of illness and death worldwide. Mosquitoes are primary vectors for many dreadful and fatal diseases such as dengue, malaria, yellow fever and filariasis. It can transmit diseases to more than 700 million people each year^{8,33}. Lymphatic filariasis infects 120 million people in 73 countries worldwide and continues to be a worsening problem, especially in Africa and the Indian subcontinent²⁸. Control of such diseases is becoming increasingly difficult because of increasing resistance to synthetic insecticides²⁹.

Schistosomiasis affects more than 200 million people worldwide and is considered as the world's most widespread parasitic disease¹⁹. The life cycle of the parasite in Egypt includes *Biomphalaria* and *Bulinus* snails as intermediate hosts¹². Interruption of the parasite's life cycle, via control of the snail's population, is one of the strategies to combat schistosomiasis³⁸. Molluscicidal agents thus interrupt the life cycle of the causative parasite and prevent human infection²¹.

Using natural products of plant origin (botanical derivatives) is an alternative and recent approach for mosquito and snail control. Despite their toxicity to pests and snails, they are readily biodegradable and usually lack toxicity to higher animals so they are eco-friendly^{4,5}. Essential oils of plants are outstanding candidates, since they are; in some cases;

highly active, readily available in tropical countries and economically viable²¹. Essential oils molluscicidal and mosquitocidal activities have been reported by many studies^{23,24,27}.

Thymus capitatus Hoff. et Link. and *Marrubium vulgare* L. are aromatic plants belonging to the family Lamiaceae. They are distributed along the Mediterranean area, at the North coast of Egypt. Few reports dealt with mosquitocidal activity of *T. vulgaris*^{7,18} and nothing was traced concerning the mosquitocidal activity of *M. vulgare* or the molluscicidal activity of both plants. In this respect, the present work is an attempt to characterize the different constituents of oils hydro distilled from *T. capitatus* and *M. vulgare* and to evaluate the effect of these essential oils of both plants on *B. alexandrina* snails and their egg masses as well as on larvae and pupae of *Culex pipiens*.

MATERIAL AND METHODS

Plant material: The aerial parts of *T. capitatus* and *M. vulgare* were collected from the North coast of Egypt during April 2010. Authentication of the plant was established in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt. Voucher specimens (No. T-12 and M-22).

Preparation of the essential oil: Fresh aerial parts (500 g) of both

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plants under investigation were separately subjected to hydrodistillation (HD) in a Clevenger-type apparatus and the oil obtained from each plant was dried over anhydrous sodium sulfate and stored in a refrigerator till analysis. The percentage yield for each sample was determined. The specific gravity and refractive index for each oil sample were also determined¹³.

Analysis of the oils: Investigation of the prepared oils was carried out on an Agilent (USA) Gas Chromatography-Mass Spectrometry system (GC-MS), model 6890, fitted with an Agilent mass spectroscopic detector (MSD), model 5937, as well as a 30 m long, cross-linked 5% phenyl polysiloxane (HP-5MS, Hewlett Packard, USA) fused-silica column (i. d. 0.25 mm, film thickness 0.25 µm). The initial temperature was 80 °C, kept isothermal for three min, then increased to 260 °C at 8 °C/min, and the final temperature was kept isothermal for 15 min. The ion source temperature was 230 °C and the quadrupole temperature was 150 °C. The

carrier gas was helium adjusted at a flow rate of 0.1 mL/min. Ionization energy was 70 eV, and scan range was 40 - 500 *m/z* at 3.62/scan.

Identification of the oil components: Search for identification of the oil components was carried out using the Willey 275 L GC-MS library data base. A series of authentic *n*-alkanes (C8-C22, Poly Science Inc., Niles, USA) was subjected to Gas Liquid Chromatography (GLC) analysis under the same experimental conditions. The retention indices (Kovats' indices, KI) of the volatile constituents were computed by logarithmic interpolation between bracketing alkanes¹⁷. Identification of the individual components was confirmed by comparison of their retention indices and MS fragmentation patterns with published data². Relative percentages were calculated from the Total Ion Chromatograms by the computerized integrator. Results of GC/MS analysis and for the relative percentages are shown in Tables 1 and 2 respectively.

Table 1
Results of GC/MS analysis of the essential oil of *Thymus capitatus* Hoff. et Link. and *Marrubium vulgare* L.

No.	Rt (min.)	KI	Identified compound	Percentage	
				<i>T. capitatus</i>	<i>M. vulgare</i>
1	9.08	1167	Borneol	9.15	-----
2	11.24	1293	Thymol	32.82	34.55
3	11.67	1298	Carvacrol	32.98	4.35
4	12.1	1335	δ-Elemene	-----	2.16
5	12.37	1347	α-Terpinyl acetate	-----	0.51
6	12.41	1355	Thymol acetate	3.27	-----
7	12.71	1368	Carvacrol acetate	1.8	-----
8	12.98	1403	Caryophyllene	6.15	-----
9	13.42	1431	γ-Elemene	-----	1.24
10	13.45	1443	Aromandrene	0.43	-----
11	13.72	1451	α-Humulene	0.3	1.89
12	13.80	1479	Germacrene D	-----	0.74
13	13.90	1480	α-Amorphene	-----	2.39
14	13.93	1509	γ-Cadinene	-----	17.68
15	13.98	1516	δ-Cadinene	0.38	2.21
16	14.05	1525	α-Cadinene	-----	2.97
17	14.12	1559	α-Muurolene	-----	1.20
18	14.45	1566	Germacrene D-4-ol	-----	6.37
19	15.90	1568	Spathulenol	1.2	-----
20	15.96	1580	Caryophyllene oxide	3.45	1.74
21	16.75	1636	Caryophylla-4(14),8,(15)-diene-5-β-ol	1.17	2.55
22	17.03	1640	Alloaromadendrene	1.01	-----
23	17.32	1652	α-Cadinol	-----	5.39
24	17.41	1671	α-Bisabolol	0.64	-----
25	18.12	1740	Cucurmenol	-----	0.95
26	18.76	1768	Pentadecanol	0.2	1.09
27	20.33	1881	<i>n</i> -Hexadecanol	0.53	-----
28	22.25	2015	<i>n</i> -Heneicosane	0.48	-----
29	24.39	2292	<i>n</i> -Tricosane	0.31	0.21

Rt, retention time; KI, Kovats' index.

Table 2

Percentages of the different classes of constituents identified by GC/MS in the essential oils of *Thymus capitatus* Hoff. et Link. and *Marrubium vulgare* L.

Class	Percentage	
	<i>T. capitatus</i>	<i>M. vulgare</i>
Hydrocarbons	8.05	32.69
• Sesquiterpenes	7.26	32.48
• Non-terpenoid hydrocarbons	0.79	0.21
Oxygenated constituents	88.22	57.5
• Monoterpenes	9.15	0.51
• Sesquiterpenes	7.67	18.09
• Other oxygenated constituents	71.4	38.9

Collection and laboratory maintenance of snails: *Biomphalaria alexandrina* snails and egg masses were collected and identified from irrigation canals in Giza Governorate⁹. Snails were screened for natural infection with larval trematodes. Uninfected snails were maintained in the laboratory conditions for seven days before being used in the study in dechlorinated tap water and fed daily on green lettuce. Tests were carried out at room temperature (26 +1 °C). In each step, a fine mesh was placed over the container to prevent snails crawling out of the container.

Molluscicidal bioassay: The bioassay of molluscicidal activity against the *B. alexandrina* was evaluated according to the established procedures³⁶. Five adult snails (8-14 mm in diameter) and snail's egg masses (three days old) were placed, separately, in a beaker containing 200 mL of essential oil water solution of *T. capitatus* and *M. vulgare* at a series of concentrations ranging from 75-1000 parts per million (ppm) for each tested plant oil. Each experiment was set in triplicate. Snails and egg masses remained in dechlorinated water during the experiment served as control group. Immersion technique was adopted according to WHO³⁵. Adult snails and egg masses were exposed for 24h at room temperature. After 24h, snails were rinsed twice with aerated tap water. At the end of this period; tested snails and egg masses were examined to assess mortality. Mortality was evaluated using crushing technique (5% sodium hydroxide solution)³⁵. Egg masses were examined under the microscope for detecting the embryos and its vitality.

Snails were considered dead if they remained motionless, did not respond to the presence of food or if the shell looked discolored. The number of dead snails was expressed as percent mortality. The lethal concentration to 50% (LC₅₀) and 90% (LC₉₀) for snails and 100% (LC₁₀₀) for the eggs was calculated following the method of Finney¹⁵. Samples that caused no mortality at 1000 ppm were considered inactive and were not investigated.

Mosquitocidal bioassay: Eggs of *C. pipiens* were obtained from The Medical Research Institute of Insects, Giza, Egypt were soaked in dechlorinated tap water to develop into first instar larvae. Larvae were reared in the same aquarium until the development of third instar larvae and pupae. Twenty *C. pipiens* third instar larvae as well as pupae were picked up from the aquarium and located in a 200 mL beaker. The bioassay was done according to WHO guidelines with slight modifications³⁷. Essential oils of both *T. capitatus* and *M. vulgare* were

tested at the same concentrations as those applied for snails for each tested plant. Stock solution of the essential oil was prepared in Tween 80. From this stock solution, concentrations of 12.5, 25, 50, 100 and 200 ppm were prepared and replicated three times for each concentration. Mosquitoes were exposed to essential oils for 24h at room temperature, and were kept under normal laboratory conditions at 26 ± 2 °C and 60 ± 10% relative humidity with 12:12 D/L photoperiod. Mortality was recorded after 24 hours of continuous exposure during which no food was offered to the test organisms. The LC₅₀ and LC₉₀ of tested plants, 95% confidence interval and their slopes of probit regression line were determined to probit analysis program to compare their effectiveness³⁰.

RESULTS

Steam distillation of the essential oils of *T. capitatus* and *M. vulgare* yielded 0.5% and 0.2%, respectively. The specific gravity and refractive index (at 25 °C) for *T. capitatus* were 0.8561 and 1.5213, respectively while those of *M. vulgare* were 0.9562 and 1.6705, respectively. Results of GC/MS analyses of the two samples are displayed in Table 1. Numbers of the identified components in both oils were 18 and 19, amounting to 96.27% and 90.19% of the total oil composition for *T. capitatus* and *M. vulgare* respectively. Constituents identified under the adopted operating conditions of the essential oils under investigation were 29. Detected components in both samples *viz.*, thymol, carvacrol, δ-cadinene, caryophyllene oxide, caryophylla-4(14), 8,(15)-diene-5-β-ol, *n*-tricosane, pentadecanol and humulene. The rest of constituents appeared, however, unevenly distributed in the analyzed oils.

The identified amount of oxygenated constituents were 88.22% and 57.50% for *T. capitatus* and *M. vulgare*, respectively (Table 2) while the amount of identified hydrocarbons was 8.05% and 32.69%, respectively. The overall chromatographic profile of the two oil samples was dominated by the oxygenated constituents. These were mainly composed of phenols among which carvacrol (32.98%) and thymol (32.82%) were the major constituents in *T. capitatus* oil while in *M. vulgare* oil thymol (34.55%) was the major constituent. Borneol, bicyclic monoterpenoid alcohol, was only present in *T. capitatus* oil (9.15%). Sesquiterpenes were the major class of hydrocarbons in the two oil samples amounted to 7.26% and 32.48% while non-terpenoid hydrocarbons were only 0.79% and 0.21% for *T. capitatus* and *M. vulgare* oils, respectively. The major sesquiterpene hydrocarbon in *T. capitatus* was *z*-caryophyllene (6.15%) while γ-cadinene was the major sesquiterpene hydrocarbon in *M. vulgare* oil (17.68%).

Molluscicidal activity of *T. capitatus* and *M. vulgare* essential oils against adult *B. alexandrina* and egg masses was evaluated at different concentrations ranging from 75-1000 ppm. After screening; the LC₅₀ and LC₉₀ of *T. capitatus* essential oil versus adult snails were 200 and 400 ppm/3hrs, respectively while of *M. vulgare* essential oil were 50 and 100 ppm/3hrs, respectively (Table 3). Furthermore, *M. vulgare* oil showed 100% snail ovicidal activity against *B. alexandrina* eggs at 200 ppm/24 hrs. However, *T. capitatus* oil showed no snail ovicidal activity (Table 3).

Concerning mosquitocidal activity, both *T. capitatus* and *M. vulgare* oils gave 50% and 90% larvicidal activity at 100 and 200 ppm/12hrs, respectively. Moreover, both samples showed 50% and 90% pupicidal activity at 200 and 400 ppm/12hrs, respectively (Table 4).

Table 3

Results of molluscicidal activity of essential oils of *Thymus capitatus* Hoff. et Link. and *Marrubium vulgare* L. against *Biomphalaria alexandrina* after 3hr exposure period

Essential oil	Adult snails		Eggs
	LC ₅₀	LC ₉₀	LC ₁₀₀
<i>T. capitatus</i>	200 ppm	400 ppm	No effect
<i>M. vulgare</i>	50 ppm	100 ppm	200ppm*

* After 24 hr

Table 4

Results of larvicidal activity of the essential oils of *Thymus capitatus* Hoff. et Link. and *Marrubium vulgare* L. against *Culex pipiens* after 12 h exposure period

Essential oil	Larvae		Pupae	
	LC ₅₀	LC ₉₀	LC ₅₀	LC ₉₀
<i>T. capitatus</i>	100 ppm	200 ppm	100 ppm	200 ppm
<i>M. vulgare</i>	200 ppm	400 ppm	200 ppm	400 ppm

DISCUSSION

Among the most promising advances in the field of drug development is discovering new molecules or novel uses of the already available compounds with known safety and without any side effects. Thymol is a naturally occurring phenolic monoterpene, known for its antioxidant, anti-inflammatory, antimicrobial, antileishmanial, antimalarial, antiprotozoal, insecticidal and molluscicidal activities^{6,14}. Phenolic compounds were proved to be useful in a variety of molluscicidal applications²¹. Thymol showed also considerable molluscicidal effect against *B. alexandrina*, *Bulinus truncatus* and *Lymnaea natalensis*¹⁴. An isomer of thymol, namely carvacrol, showed *in vitro* antifilarial activity but to a lesser extent than thymol²⁵. Furthermore, some studies revealed that carvacrol has an antibacterial, antifungal, antiparasitic and antioxidants activities³¹. According to these results, two members of the Lamiaceae cultivated in Egypt (*T. capitatus* and *M. vulgare*) were screened for the presence of thymol in their essential oils. Both oils contain thymol in high content so they are screened for their molluscicidal and insecticidal activities as a way to relate these activities with the previous findings of thymol itself.

Both oils showed promising molluscicidal activity against *B. alexandrina* snails and mosquitocidal activity versus *C. pipiens* larvae and pupae. These activities may be attributed to thymol or its isomer carvacrol which agreed with other studies³². Volatile oils caused significant behavioral changes in snails with the most obvious sign of distress being muscular and spiral twisting of the body followed by crawling on one another. The nature and rapid onset of these responses showed that these oils probably contain neurotoxins that might be active at the neuromuscular system of exposed animals³⁹. Similar metabolic disorders on life were revealed including egg laying, egg hatchability, hepatic cells damages, lack of smooth transmission at nerve junction, loss of muscular coordination and convulsions, then snails' death¹.

The results of the present study might be attributable also to the high sesquiterpene content of *Marrubium* (50.57%) as compared to oil of *Thymus* (14.93%). Sesquiterpenoids are credited with various biological actions. They may kill snails as well as snail egg masses via contact poisoning resulting in killing the early egg embryos¹¹. These findings are in accordance with other studies that mentioned sesquiterpenes as promising skin penetration enhancers with specific P-glycoprotein modulators that can reverse cellular multidrug resistance by inhibiting the drug efflux process^{26,34}. Other studies also stated that antimicrobial properties of essential oils from *M. vulgare* are associated with their high contents of oxygenated compounds (46.21%)⁴¹. Researchers also reported mono and sesquiterpenoids as the major components of essential oils which are phenolic in nature⁴⁰. It is therefore reasonable to assume that their antimicrobial activity might be related to the abundance of phenolic compounds²¹.

It was reported that methanolic extracts from many plants had the maximum larvicidal activity against *C. pipiens* larvae; moreover, plant extracts have been shown to have good effective control properties on mosquitoes besides being environmental friendly bio-pesticide²⁰. On the contrary, other studies revealed that methanolic extracts were less effective than the extracts of other essential oils³. Researchers suggested that larvicides affect mosquito in one of three possible mechanisms; by physical flooding of the tracheal system or by toxicity specially that of volatile components and by interference with surface forces¹⁰. Different sensitivities of mosquito species towards different volatile oils has been also recorded³. Thymol, one of the major components of *Lippia sidoides*, was identified as an active component of the larvicidal action against *Aedes aegypti* causing 100% larval mortality¹⁶.

In conclusion, the essential oils of both *M. vulgare* and *T. capitatus* are biologically active agents against mosquitoes and snails, and effect showed significant time and dose dependant. Our results suggest that the essential oils of these plants may have a promising role in this regard.

RESUMO

Atividades moluscicida e mosquitocida de óleos essenciais de *Thymus capitatus* Hoff. et Link. e de *Marrubium vulgare* L.

A destilação por arraste a vapor dos óleos essenciais de partes aéreas de *Thymus capitatus* Hoff. et Link. e de *Marrubium vulgare* L. coletadas na costa norte do Egito resultaram em rendimento de 0,5% e 0,2%, respectivamente. Resultados de análises por cromatografia gasosa acoplada à espectrometria de massas de ambas as amostras possibilitaram a identificação de 96,27% e 90,19% dos constituintes químicos respectivamente de *T. capitatus* e *M. vulgare*. Verificou-se predomínio de constituintes oxigenados (88,22% para *T. capitatus* e 57,50% para *M. vulgare*, principalmente fenóis, como carvacrol (32,98%) e timol (32,82%) no óleo essencial de *T. capitatus*, e timol (34,55%) no óleo essencial de *M. vulgare*. Avaliou-se a atividade dos óleos essenciais obtidos contra adultos e ovos de *Biomphalaria alexandrina*, bem como em larvas e pupas de *Culex pipiens*. A CL₅₀ e CL₉₀ do óleo essencial de *T. capitatus* em moluscos adultos foi respectivamente 200 e 400 ppm/3hrs, enquanto para o óleo essencial de *M. vulgare* verificou-se CL₅₀ e CL₉₀ de 50 e 100 ppm/3hrs, respectivamente. Além disso, *M. vulgare* apresentou atividade ovicida, com CL 100 de 200 ppm/24 horas, enquanto o óleo essencial de *T. capitatus* não demonstrou atividade ovicida. Verificou-se ainda atividade mosquitocida,

com CL₅₀ e CL₉₀ de 100 e 200 ppm/12hrs respectivamente para larvas, e 200 e 400 ppm/12hrs contra pupas de *C. pipiens*.

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