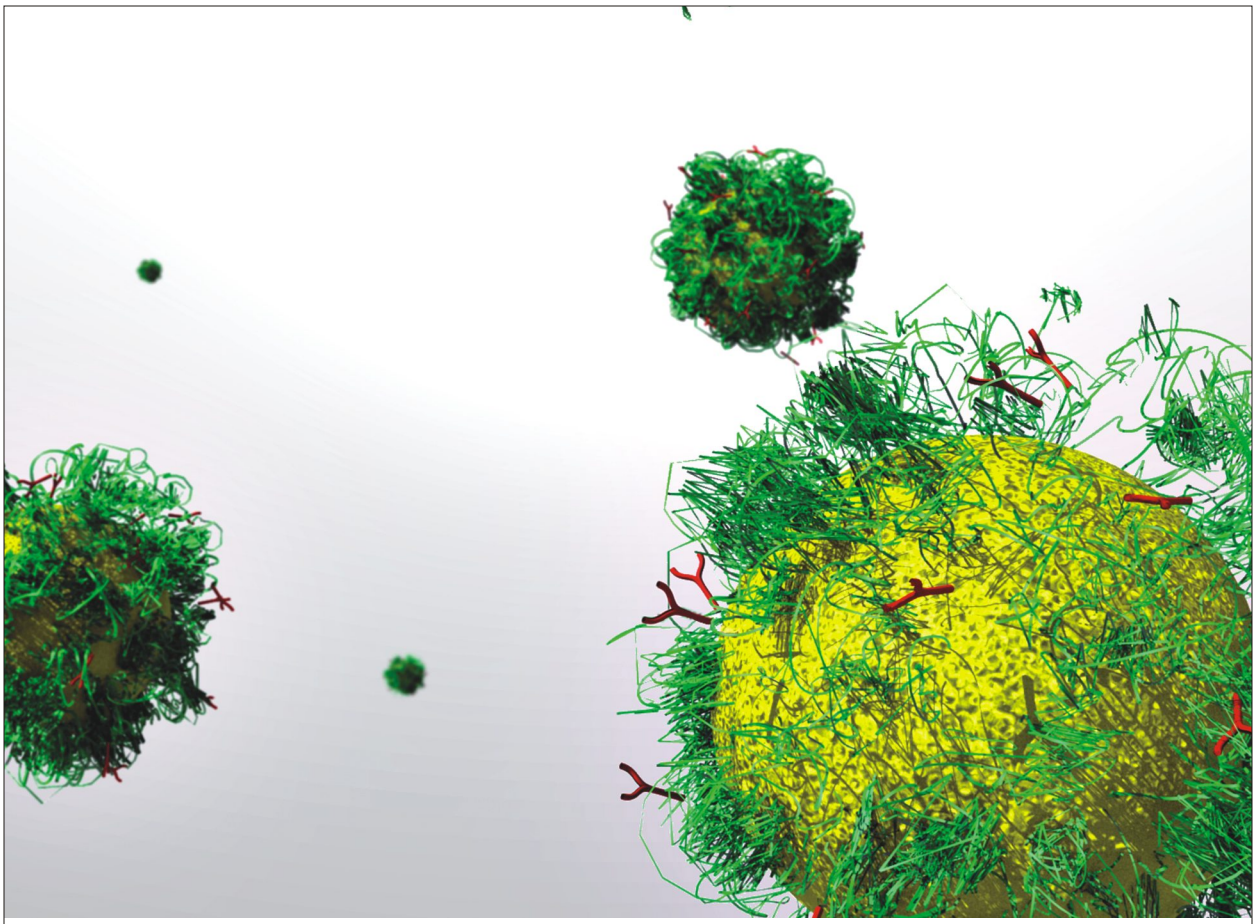

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Molluscicidal and Mosquitocidal Activities of the Essential oils of *Thymus capitatus* L. and *Marrubium vulgare* L.

[1]M.M. Salama, [2]E.E. Taher and [3]M.M. El. Bahy
[1]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr el-Aini street, 11561, Cairo, Egypt
[2]Department of Parasitology, Research Institute of Ophthalmology, Giza, Egypt
[3]Department of Parasitology, Faculty of Veterinary Medicine, Cairo University, Egypt

Corresponding Author: M.M. Salama, Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr el-Aini Street, 11561, Cairo, Egypt

ABSTRACT

Molluscicidal activity of *Thymus capitatus* and *Marrubium vulgare* essential oils on adult and eggs of *Biomphalaria alexandrina* as well as on different stages of *Culex pipiens* was evaluated for their effectiveness on vector control. Steam distillation of essential oils of the flowering aerial parts of both *Thymus capitatus* L. and *Marrubium vulgare* L. yielded 0.5 and 0.2%, respectively. Results of GC/MS analyses of the two samples revealed an identified components in both oils amounted to 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 and 57.50% for *T. capitatus* and *M. vulgare,* respectively). 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 was present only in *T. capitatus* oil (9.15%). *T. capitatus* essential oil gave LC_{50} and LC_{90} mortality against adult snails at 200 and 400 ppm/3 h, respectively while that of *M. vulgare* was 50 and 100 ppm/3 h, respectively. On the other hand, *M. vulgare* showed LC_{100} ovicidal activity at 200 ppm/24 h while *T. capitatus* oil showed no ovicidal activity. Insecticidal activity of both two essential oils revealed LC_{50} and LC_{90} larvicidal activity at 100 and 200 ppm/12 h, respectively and LC_{50} and LC_{90} pupicidal activity at 200 and 400 ppm/12 h, respectively. Results of this study suggest that plant essential oils may have a promising role in vector control with needed continuing investigations.

Key words: Plant extracts, *Thymus capitatus*, *Marrubium vulgare*, molluscicides, *Biomphalaria alexandrina*, larvaecides, *Culex pipiens*

INTRODUCTION

Vector borne diseases are a major source of illness and death worldwide. More than 700 million people worldwide are affected by fatal diseases transmitted through mosquito vectors such as malaria, dengue, filariasis (Taubes, 1997; Cetin *et al.*, 2010). *Culex pipiens* transmits filariasis to more than 120 million people in 73 countries constituting a big problem (Ottesen *et al.*, 1997). Mosquito control is becoming an increasing problem due to resistance to synthetic insecticides (Ranson *et al.*, 2001).

Schistosomiasis is a widespread parasitic disease affecting more than 200 million people worldwide (King and Dangerfield-Cha, 2008). It is an important disease in Egypt and in many other tropical countries. The disease is transmitted through an intermediate host *Biomphalaria* and *Bulinus* snails (Davis, 1996). One of the strategies to combat schistosomiasis is to interrupt the parasite's life cycle in endemic areas via control of the snail's population (its intermediate host) (WHO, 1985). Destroying the snails using molluscicidal agents is one way to interrupt the life cycle of the causative parasite and to prevent human infection (Lahlou, 2004).

The use of botanical derivatives is an alternative and recent approach for mosquito and snail control. Beside their toxicity to pests and snails, they are readily biodegradable and usually lack toxicity to higher animals that means they are eco-friendly (Bowers, 1992; Al-Zanbagi *et al*., 2001).

Essential oils of plants are highly active, readily available candidates in tropical countries and economically viable (Lemos *et al*., 1992). Studies of the essential oil molluscicidal and mosquitocidal activities have been reported (Khallouki *et al*., 2000; Youssif and Shaalan, 2001; EL-Kamali *et al*., 2010; Oparaocha *et al*., 2010). *Thymus capitatus* L and *Marrubium vulgare* L. are aromatic plants belonging to the family Lamiaceae. They are distributed along the Mediterranean area, at the north coast, in Egypt. Few reports dealt with mosquitocidal activity of *Thymus vulgaris* (Mansour *et al*., 2000) and nothing was traced concerning the mosquitocidal activity of *M. vulgare* or the molluscicidal activity of both plants. In that respect, the present study is an attempt to characterize the different constituents of oils extracted from *Thymus capitatus* L. and *Marrubium vulgare* L. Moreover, to evaluate the dual effect of these volatile oils of both plants on both *Biomphalaria alexandrina* snails and their egg masses as well as on *Culex pipiens* larvae and pupae stages.

MATERIAL AND METHODS

Plant material: The aerial parts of *T. capitatus* L. and *M .vulgare* L. were collected from the north coast of Egypt, during April 2010. Authentication of the plant was established by Dr. Mohammed El-Gebaly, Senior botanist, Cairo, Egypt. Voucher specimens (No. T-12 and M-22) are kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt.

Preparation of the essential oil: Fresh aerial parts (500 g) of both plants under investigation were subjected to Hydrodistillation (HD) in a Clevenger-type apparatus (Egyptian Pharmacopeia, 2005). 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 using an Abbe refractometer Lecia Mark II, RL. 19E-10846-5 for each oil sample was also determined.

Analysis of the oils: Investigation of the prepared oils was carried out on an Agilent (USA) GC-MS system, 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 3 min, then increased to 260°C at 8°C min^{-1} 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^{-1}. Ionization energy was 70 eV and scan range was 40-500 m z^{-1} at 3.62 $scan^{-1}$.

Table 1: Results of GC/MS analysis of the volatile oil of *T. capitatus* and *M. vulgare*

			Percentage	
Rt (min.)	KI	Identified compound	*T. capitatus*	*M. vulgare*
9.08	1167	Borneol	9.15	----------
11.24	1293	Thymol	32.82	34.55
11.67	1298	Carvacrol	32.98	4.35
12.1	1335	δ-Elemene	---------	2.16
12.37	1347	α-Terpinyl acetate	---------	0.51
12.41	1355	Thymol acetate	3.27	---------
12.71	1368	Carvacrol acetate	1.8	---------
12.98	1403	Caryophyllene *Z*	6.15	---------
13.42	1431	γ-Elemene	-------	1.24
13.45	1443	Aromandrene	0.43	--------
13.72	1451	α-Humulene	0.3	1.89
13.80	1479	Germacrene D	--------	0.74
13.90	1480	α-Amorphene	-----	2.39
13.93	1509	γ-Cadinene	------	17.68
13.98	1516	δ-Cadinene	0.38	2.21
14.05	1525	α-Cadinene	---------	2.97
14.12	1559	α-Muurolene	--------	1.20
14.45	1566	Germacrene D-4-ol	--------	6.37
15.90	1568	Spathulenol	1.2	---------
15.96	1580	Caryophyllene oxide	3.45	1.74
16.75	1636	Caryophylla-4(14),8 (15)-diene-5-β-ol	1.17	2.55
17.03	1640	Epoxy-allo-alloaromadendrene	1.01	------
17.32	1652	α-Cadinol	---------	5.39
17.41	1671	α-Bisabolol	0.64	------
18.12	1740	Cucurmenol	--------	0.95
18.76	1768	Pentadecanol 2	0.2	1.09
20.33	1881	*n*-Hexadecanol	0.53	-------
22.25	2015	*n*-Heneicosane	0.48	-------
24.39	2292	*n*-Tricosane	0.31	0.21
Total identified compounds			96.27	90.19

Rt: Retention time, KI: Kovats index

Identification of the oil components: Library search for identification of the oil components was carried out using a Willey and Nist (6th ed.) 275 L GC-MS data base. A series of authentic n-alkanes (C8-C22, Poly Science Inc., Niles, USA) was subjected to GLC analysis under the same experimental conditions. The retention indices (Kovats indices, KI) of the volatile constituents were computed by logarithmic interpolation between bracketing alkanes (Egyptian Pharmacopeia, 2005). Identification of the individual components was confirmed by comparison of their retention indices and MS fragmentation patterns with published data (Adams, 2005). Relative percentages were calculated from the Total Ion Chromatograms by the computerized integrator. The results of GC/MS analysis are recorded in Table 1.

Snails' collection and maintenance: Biomphalaria alexandrina snails were collected from irrigation canals in Giza Governorate. Snails were identified according to Christensen and Frandsen (1985). Snails were screened for natural infection with any trematodes. Uninfected snails were maintained in the laboratory conditions for seven days before being used in our molluscicidal

tests in de-chlorinated tap water and fed daily on green lettuce. Tests were carried out at room temperature (26+1°C). In each step, snails were prevented from crawling out of the container by means of a fine mesh placed above the water surface.

Molluscicidal bioassay: The bioassay of molluscicidal activity against the snails *Biomphalaria alexandrina* was evaluated according to the established procedures (WHO, 1965). Five adult snails (8-14 mm in diameter) and snail's egg masses (3 days old) were placed, separately, in a beaker containing 200 mL of essential oil water solution of *Thymus capitatus* and *Marrubium vulgare* at a series of concentrations ranging from (75-1000 ppm) for each tested plant. The following concentrations were evaluated (200, 100, 50, 25 and 12.5 mg). Each experimental concentration was set in triplicate. Immersion technique was adopted according to WHO (1961). Adult snails and egg masses were exposed for 24 h at room temperature and were kept under normal diurnal lighting. After 24 h, the snails were rinsed twice with aerated tap water. At the end of this period the tested snails and egg masses were examined to assess mortality. Mortality was evaluated using crushing technique (5% sodium hydroxide solution) (WHO, 1961). Egg masses were examined under the microscope detecting the developmental stages of their 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. In control experiment, snails and egg masses were not exposed to the potential extract and these remained in de-chlorinated water during the experiment. The number of dead snails was expressed as % mortality. The concentration lethal to 50% (LC_{50}) and 90% (LC_{90}) was calculated following the method of Finney (1972). Samples that cause no mortality at (1000 ppm) were considered inactive and were not investigated.

Larvicidal bioassay: The vector mosquito, *Culex pipiens*, was used as test organism. Mosquito eggs were obtained from The Medical Research Institute of Insects. Eggs were soaked in de-chlorinated tap water to develop into first instar larvae. Larvae were reared in the same aquarium till development of third instar larvae and pupae. In a 200 mL beaker; 20 third instar larval stage as well as pupa of *C. pipiens* were picked from the aquarium. The bioassay was done according to WHO (1981) guidelines with slight modifications. The volatile oils of both *T. capitatus* and *M. vulgare* were tested at the same concentrations as those applied for snails for each tested plant, dilution of the essential oil were prepared in Tween 80 to provide a stock solution. 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 24 h at room temperature, and were kept under normal laboratory conditions at ±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 concentration lethal to 50% (LC_{50}) and 90% (LC_{90}) of test organisms, 95% confidence interval and their slopes of probit regression line were determined to probit analysis program to compare their effectiveness (Raymond, 1985).

RESULTS AND DISCUSSION

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 and 2. The number of the identified components in both oils was 18 and 19, amounting to 96.27 and 90.19% of the total oil composition for *T. capitatus* and *M. vulgare*, respectively.

Table 2: The relative percentages of the different classes of constituents identified in the essential oils of *T. capitatus* and *M. vulgare*

	Percentage	
Class	*T. capitatus*	*M. vulgare*
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

Table 3: Results of molluscicidal activity of essential oils of *T. capitatus* and *M. vulgare*

	Adult snails	Eggs	
Essential oil	LC_{50}	LC_{90}	LC_{100}
T. capitatus	200 ppm/3 h	400 ppm/ 3 h	-----
M. vulgare	50 ppm/3 h	100 ppm/ 3 h	200 ppm/24 h

The total number of constituents identified under the adopted operating conditions of the essential oils under investigation was 29 among which 6 components viz., thymol, carvacrol, δ-cadinene, caryophyllene oxide, caryophylla-4(14), 8 (15)-diene-5-β-ol, n-tricosane were detected in both samples. The rest of constituents appeared, however, unevenly distributed in the analyzed oils.

The amount of identified oxygenated constituents Table 2 were 88.22 and 57.50% for *T. capitatus* and *M. vulgare,* respectively while the amount of identified hydrocarbons were 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 was 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%).

The molluscicidal activity of *T. capitatus* and *M. vulgare* essential oils against adult *Biomphalaria alexandrina* and egg masses was evaluated at different concentrations ranging from (75-1000 ppm). After screening, the LD_{50} (lethal dose of 50% mortality) and LD_{90} (lethal dose of 100% mortality) were determined. *T. capitatus* essential oil gave 50 and 90% mortality for adult snails at doses of 200 and 400 ppm/3 h, respectively while *M. vulgare* essential oil gave 50 and 90% mortality against adult snails at doses 50 and 100 ppm/3 h, respectively Table 3. Furthermore, the oil of *M. vulgare* showed 100% snail ovicidal activity at 200 ppm. 24 h against the eggs of *Biomphalaria alexandrina* however, that of *T. capitatus* showed no snail ovicidal activity Table 3. The results obtained might be attributable to the high sesquiterpene content of

Table 4: Results of mosquitocidal activity of the essential oils of *T. capitatus* and *M. vulgare*

	Larva		Pupa	
Essential oil	LC_{50}	LC_{90}	LC_{50}	LC_{90}
T. capitatus	100 ppm/12 h	200 ppm/12 h	100 ppm/12 h	200 ppm/12 h
M. vulgare	200 ppm/12 h	400 ppm/12 h	200 ppm/12 h	400 ppm/12 h

marrubium (40.57%) as compared to thyme oil (14.93%) (Table 2). Sesquiterpenoids are credited with various biological actions. In the author's opinion, sesquiterpene compounds may kill snails via contact poisoning. This effect was also proved on snails' egg masses resulting in killing the early egg embryos. These data were in agreement with Cornwell and Barry (1994) and Santoro *et al.* (2007) who mentioned that, sesquiterpenes are promising skin penetration enhancers. Munoz-Martinez *et al.* (2004) revealed also that sesquiterpenes have specific P-glycoprotein modulators that can reverse cellular multidrug resistance by inhibiting the drug efflux process .

Concerning mosquitocidal activity of *T. capitatus* and *M. vulgare* essential oils were tested against larvae and pupae stages of *Culex pipiens*. Evaluation was performed at different concentrations ranging from (75-1000 ppm) to obtain the LD_{50} (lethal dose of 50% mortality) and LD_{90} (lethal dose of 100% mortality). Both *T. capitatus* and *M. vulgare* oils gave 50 and 90% larvicidal activity at 100 and 200 ppm/12 h, respectively. Moreover, both samples showed 50 and 90% pupicidal activity at 200 and 400 ppm/12 h, respectively Table 4.

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 (Braga *et al.*, 2006a), anti-inflammatory (Braga *et al.*, 2006b), antimicrobial (Khanuja *et al.*, 2004), antileishmanial, antimalarial, antiprotozoal (Tasdemir *et al.*, 2006), insecticidal and molluscicidal activities (El-Din, 2006). Phenolic compounds were proved to be useful in a variety of molluscicidal applications (Lahlou, 2004). Thymol showed also considerable molluscicidal effect against *Biomphalaria alexandrina*, *Bulinus truncatus* and *Lymnaea natalensis* (El-Din, 2006). Thymol was also screened to be the active principle responsible for antifilarial activity. An isomer of thymol, namely carvacrol, also showed *in vitro* antifilarial activity but to a lesser extent than thymol (Mathew *et al.*, 2008). This was also revealed in some studies that carvacrol has an antibacterial, antifungal, antiparasitic and antioxidants activities (Singh *et al.*, 2011). According to these results, two members of the family 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 subjected to be 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 *Biomphalaria alexandrina* snails and insecticidal activity on both larvae and pupae stages of *Culex pipiens* and these activities may be attributed to thymol or its isomer carvacrol. 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 animals. This was also revealed by other studies that on applying plant molluscicide; results reflect the effect of the metabolic disorders on life, egg laying, egg hatchability, hepatic cells damages, lack of smooth transmission at nerve junction, loss of muscular coordination and convulsions, then snails' death (Abdel Kader *et al.*, 2005).

CONCLUSION

Oil extracts of both marrubium and thyme which are Egyptian native plants continue to provide a wealth of potential sources for biologically active agents that may be applied against arthropod pests of man and animals. Present results suggest that plant essential oils may have a promising role in this regard and are continuing these investigations in our laboratory.

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