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Effects of sublethal doses of selected botanical molluscicides on oxygen consumption of the brown garden snail, *Eobania vermiculata*.

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Abstract

Laboratory bioassays were carried out for evaluating the efficacy of certain plant materials including Nicotine, Thymol, Menthol, Caffeine and Camphor as molluscicides against the brown garden snail, *Eobania vermiculata* using the topical application method. The obtained results proved that Nicotine and Thymol were the most promising from the molluscicidal point of view with LD_{50} 204.02 and 551.20µg/snail for the two materials, respectively. The effects of sublethal doses (LD_{25} and LD_{50}) of the most potent materials, Thymol and Nicotine, on the oxygen consumption of *E. vermiculata* snails after 1, 7 and 15 days post treatment were evaluated. Results indicated that only snails treated with LD_{50} Thymol showed significant decrease in their oxygen uptake; while all the other treatments stimulated significant increase in the oxygen consumption along the three periods of estimation. This stimulation was most pronounced with the low sublethal doses of the both examined botanical molluscicides. The succession of time post exposure did not enable the treated snails to eliminate the adverse effects of the applied materials.

Depending on the results of the current investigation, we can conclude that the both examined botanical materials, Thymol and Nicotine, are effective in killing the agricultural pest *E. vermiculata* but further studies are needed to evaluate the efficacy of these materials as safe and economic molluscicides in the field.

Keywords: Molluscicides; Thymol; Nicotine; Snails; *Eobania vermiculata;* Oxygen Consumption.

Introduction

In Egyptian fields, the terrestrial snail, *E. vermiculata*, family Helicidae, is considered as one of the most agricultural pests causing a great damage to all plant parts of different crops (**El-Okda, 1979** and **Mahrous *et al.*, 2002**).

Application of synthetic molluscicides is remaining the most effective method for controlling mollusk pests, particularly over large areas (**Radwan *et al*., 1992; Schuytema *et al,* 1994** and **Heiba *et al,* 2002**). However, their immense use has a harmful effect on non-target species and lead to pollution of the environment (**Godan, 1983**).Therefore, much effort has been focused on plant materials for potential uses as commercial pesticides in the hope that they might provide cheap, locally produced, biodegradable, environmentally safe and effective control agents.

Oxygen uptake is one of the best indicators of the overall metabolic activities of an organism (**Von Brand *et al.,* 1949**). Therefore, beside the conventional bioassay methods for testing molluscicidal action, respirometry can be used as a practical, sensitive and accurate method for the evaluation of molluscicides (**Abel-Raheem *et al.,* 1980**).
Several studies dealt with the impact of different chemical compounds on the

oxygen uptake of snails and slugs **(Bharathi and Prasada, 1989; Christian and Tesfamichael, 1990; Nair *et al.*, 1995).** To the best of our knowledge, regarding the plant origin molluscicides, very little work has been carried out on this respect **(Clark and Appleton, 1996).**The current investigation aims to study the influence of sublethal doses of two potent botanical molluscicides (Thymol and Nicotine) on the oxygen consumption of *E. vermiculata* snails at different time intervals of exposure.

Materials and methods

Collection and adaptation of snails:

Adult specimens (20-30 mm shell diameter) of the land snail, *E. vermiculata* were collected from Al-Montazah Park, Alexandria governorate during spring 2010. The collected snails were transferred in cloth sacs to the laboratory and kept in aerated cages (40×30×30cm, with 100 individuals per cage) for two weeks to acclimatize with the laboratory conditions (26-30 °C and 62±2 RH). These snails were supplied with fresh lettuce leaves three times a week.

Plant materials:

Five plant materials were used in the present study: Nicotine ($C_{10}H_{14}N_2$, MW 162.23, purity 99%), Caffeine ($C_8H_{10}N_4O_2$, MW 194.19, purity 99%), Thymol (2-[$(CH_3)_2CH$] C_6H_3-5-(CH_3)OH, MW 150.22, purity 99.5%), Menthol ($C_{10}H_{20}O$, MW 156.27, purity 99%) and Camphor ($C_{10}H_{16}O$, MW 152.23, purity 95%). They were prepared in Dimethyl Sulfoxide [DMSO] ($(CH_3)_2SO$, MW 78.13, purity 99%) which causes little distress to slugs and snails and has been shown to be the most appropriate solvent for topical application **(Young and Wilkins, 1989 ; Radwan *et al.*, 2008)**, and serially diluted with the same solvent to achieve the desired concentrations. Plant materials and DMSO were purchased from Sigma-Aldrich Company.

Bioassay:

Topical application, the method of **Hussein *et al*. (1994),** was used. Preliminary experiments were carried out to establish the effective range concentration of the tested plant materials. The tested concentrations were: 10, 15, 20, 25, 30, 40 and 50 mg/ml for Thymol, 3, 5, 7, 10, 13, 16, 20, 23 and 27 mg/ml for Nicotine, 15, 20, 30, 45, 60, 80 and 90 mg/ml for camphor, 10, 25, 40, 50, 60, 80 and 90 mg/ml for Menthol and 10, 15, 20, 25, 35, 40, 45, 50 and 60 mg/ml for Caffeine. The tested dose (30 µl) was gently applied on the surface of the snail's body inside the shell using a micropipette; while control snails were treated with DMSO. Snails were kept in plastic boxes (10×20×10cm) covered with cloth netting secured with rubber bands to prevent snails from escaping. They were fed on fresh lettuce leaves after 24 hr of treatment. The numbers of dead animals were recorded 48 hr after treatment. The test was replicated 4 times using 10 animals for each concentration.

Measurement of Oxygen consumption:

Oxygen uptake of *E. vermiculata* snails was determined using Warburg's manometric technique **(Umbreit *et al.* 1964)** using 50 ml capacity vessels each containing a single snail. CO_2 was absorbed by freshly prepared 0.4ml 10% KOH placed in the side arm with a filter paper fan (Whatman No. 1) to increase the absorbent surface. A thermobarometer consisting of an empty flask containing only KOH was included in each experiment to compensate for short-term fluctuations in atmospheric pressure. In each experiment, six flasks were submerged in a water bath maintained at 28±0.01°C and an equilibrium period of one hour was allowed before closing the stopcock of the manometer. Readings were recorded at 20-minute intervals for two-hour period. The volume of the experimental snails was determined by displacement method and their weight was measured carefully using analytical digital balance with 0.01mg accuracy (model JK-200, Komatsu, Japan). Oxygen uptake was expressed as ml O_2/g Wet wt/h.

Statistical analysis:

Percentage mortality rates were corrected using Abbott's formula (**Abbott, 1925)**; toxicity parameters for each tested plant material were determined by the probit analysis method of **Finney (1971)** on Windows® platform Version 2002. The values of oxygen consumption were represented as means ± SD and the differences were analyzed for significance by Student's (t) test using statistical analysis program, Biostat-2006 on Windows® platform Version 2002. Average stimulation (+) or inhibition (-) of oxygen consumption in percent of control was calculated according to the following equation:

$$\text{Change \%} = \frac{\text{Test -control}}{\text{Control}} \times 100$$

Results

The data obtained from examining the molluscicidal activity of the studied botanical materials showed that Thymol and Nicotine were the most potent with LD_{50} of 204.02 and 551.20µg/snail, respectively (Table 1).

The fluctuations in oxygen consumption of *E. vermiculata* snails of both the control and treated groups after 1, 7 and 15 days post exposure were represented in figures 1, 2 and 3, respectively. In comparison with the control group, treatment of snails with LD_{50} Thymol inhibited the rate of oxygen uptake along the three post exposure periods. This decrease was statistically significant at 1 and 15 days post treatment with 31.48% and 17.86% lower than the corresponding controls respectively. On contrast, all the other treatments (LD_{25} Thymol, LD_{50} and LD_{25} Nicotine) stimulated significant increase in the oxygen uptake along the three periods of estimation (Table 2).

Also the data in table 2 indicated that snails treated with low sublethal doses of both the examined botanical molluscicides exhibited the most pronounced stimulation in oxygen uptake. In addition, the treated snails were unable to eliminate the adverse effects of the applied materials throughout the whole experimental period.

Table 1.

Molluscicidal activity of selected botanical materials against *E. vermiculata* snails using the topical application method.

Botanical material	LD_{25} (µg/ snail) after 48h	LD_{50}[a] (µg/ snail) after 48h	Confidence limits for LD_{50}[b]
Thymol	377.63	551.20	491.65-609.33
Nicotine	116.17	204.02	173.32-233.47
Camphor	893.56	1354.07	1210.68-1509.42
Menthol	673.75	1252.17	1065.13-1466.92
Caffeine	524.30	932.37	822.19-1055.62

[a] *Means based on 4 replicates (n = 4), 10 animals each.*

[b] *Lower and upper Limits for LD_{50} at 95% confidence*

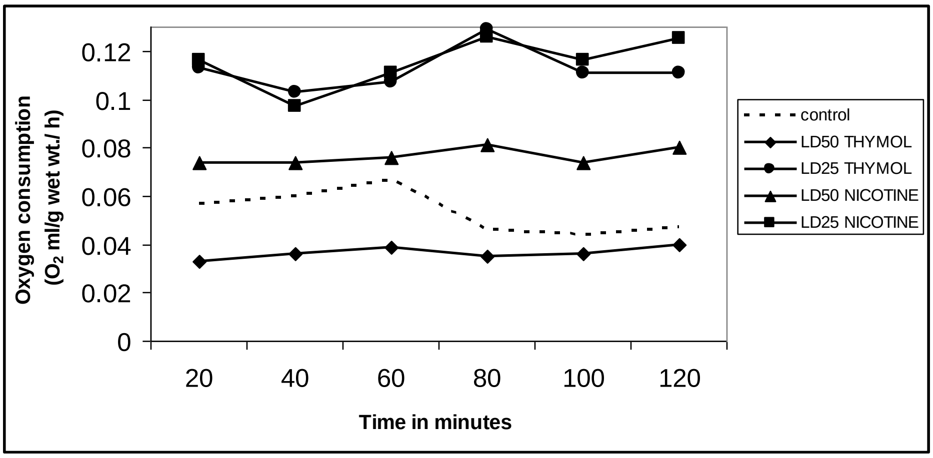

Fig 1. *Mean oxygen consumption of E. vermiculata snails post 1 day of treatment with sublethal doses of Thymol and Nicotine. Each point represents the mean value of 6 determinations.*

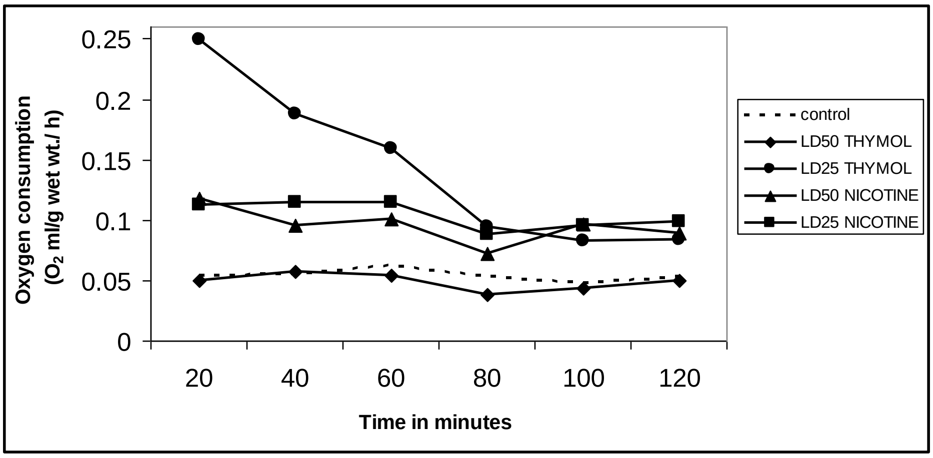

Fig 2. *Mean oxygen consumption of E. vermiculata post 7 days of treatment with sublethal doses of Thymol and Nicotine. Each point represents the mean value of 6 determinations.*

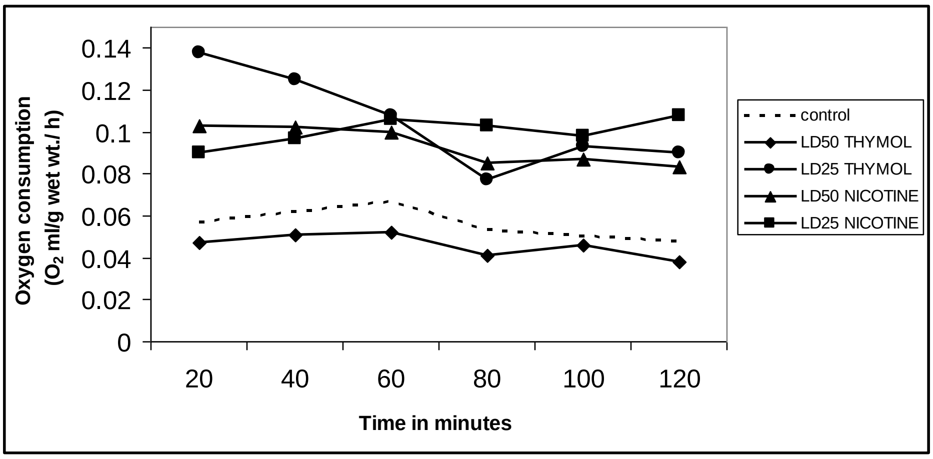

Fig 3. *Mean oxygen consumption of E. vermiculata post 15 days of treatment with sublethal doses of Thymol and Nicotine. Each point represents the mean value of 6 determinations.*

Table 2.
The effect of sublethal doses of Thymol and Nicotine on the oxygen consumption (ml/g. wet. wt./h) of *E. vermiculata* snails at three time intervals post exposure.

Time interval post exposure (Days)	Examined group	Minimum and maximum readings of oxygen consumption	Mean ± S.D.[a]	Average stimulation (+) or inhibition (-) of oxygen consumption in percent of control
1	Control	0.044-0.067	0.054±0.009	
	LD_{50} Thymol	0.033-0.040	0.037±0.003 *	-31.48
	LD_{25} Thymol	0.103-0.129	0.112±0.009 *	+107.41
	LD_{50} Nicotine	0.074-0.081	0.077±0.003 *	+42.59
	LD_{25} Nicotine	0.097-0.126	0.115±0.011 *	+112.96
7	Control	0.048-0.063	0.054±0.005	
	LD_{50} Thymol	0.038-0.057	0.049±0.007	-9.26
	LD_{25} Thymol	0.083-0.249	0.143±0.068 *	+164.82
	LD_{50} Nicotine	0.072-0.118	0.096±0.015 *	+77.77
	LD_{25} Nicotine	0.088-0.115	0.104±0.011 *	+92.59
15	Control	0.048-0.067	0.056±0.007	
	LD_{50} Thymol	0.038-0.052	0.046±0.006 *	-17.86
	LD_{25} Thymol	0.077-0.138	0.105±0.023 *	+87.50
	LD_{50} Nicotine	0.083-0.103	0.093±0.009 *	+66.07
	LD_{25} Nicotine	0.090-0.108	0.100±0.007 *	+78.57

[a] *Mean of six experiments (6 snails each) ± standard deviation.*
** Statistically significant ($p \leq 0.05$) as compared with the controls.*

Discussion

Study of the physiology of snails might yield important clues for development of chemical means of control. Theoretically, it appears likely that snails may be killed by the use of compounds interfering with cellular respiratory mechanisms (**Von Brand *et al*., 1948**). Therefore estimating the oxygen consumption as an important mean for evaluating the molluscicidal activity has been carried out by many authors (**Bharathi and Prasada, 1989; Christian and Tesfamichael, 1990; Clark and Appleton, 1996**).

Previous investigation carried out by **Kerkut and Laverack (1956)** on the respiration of *Helix pomatia* snails, family Helicidae, showed considerable short-term fluctuations in the respiratory activity. Therefore, in the current study, the oxygen uptake of *E. vermiculata* snails in response to the influence of the two potent botanical molluscicides, Thymol and Nicotine, was recorded along a period of two hours. The results obtained by the current study indicated that treatment of *E. vermiculata* snails with LD_{50} Thymol caused a marked and long lasting decline in oxygen consumption. This finding correlates with that of **Ishak and Mohamed, 1975** who reported that exposing of *Biomphalaria alexandrina* snails to sublethal concentrations of copper sulphate and Bayloscide caused a marked reduce in oxygen uptake in direct and prolonged exposure periods. Similar results were obtained by **Von Brand *et al.* (1949)** on *Australorbis glabratus* snails treated with α-nitrostilbene. Also **Sivaramakrishna *et al*. (1991)** confirmed that the rate of oxygen consumption of the freshwater snail, *Pila globosa* and the Mussel, *Lamellidens marginalis* decreased in response to both lethal and sublethal concentrations of mercury.

The interpretation of this inhibition in oxygen uptake may be attributed to one or more of the following mechanisms: firstly, the tested compound inhibited the enzyme systems involved in oxygen consumption. This postulation was supported by the study of **Von Brand *et al.*, 1949; Ishak *et al*., 1970; Bharathi and Prasada, 1989.** The second explanation returns this inhibiting effect to the cutaneous respiration that is considered the most important in meeting the oxygen requirements of pulmonates **(Alberts, 1966).** After dermal application, the primary targets for molluscicides are the epithelial cells of the skin. Therefore these molluscicides may damage the cutaneous respiration causing changes in the oxygen uptake. Finally, molluscicides may affect the oxygen carrier protein, hemocyanin, and thus inhibit the respiration process. This assumption preassumed by **Christian and Tesfamichael, 1990.**

On the other hand, significant stimulation in the respiration of *E. vermiculata* snails was recorded in response to all the other treatments, in particular the low sublethal doses, along the three periods of estimation. This result is in agreement with that of **Bharathi and Prasada, 1989** who recorded significant increase in the oxygen uptake of the freshwater snail *Thiara torulosa* in response to sublethal concentrations of three organophosphorus insecticides. The same author mentioned that the increased rate of oxygen uptake may be considered as one of the earliest symptoms of pesticide poisoning. In addition, **Clark and Appleton (1996)** indicated that low sublethal concentrations of aqueous suspensions of the plant *Apodytes dimidiate* caused significant increase in the oxygen consumption of the snail *Helisoma duryi* relative to the controls.

Detoxification processes are reactions to limit damage by toxins or to excrete them. This process includes: (i) enzymatic reactions leading to the biotransformation of chemicals by oxidation; (ii) hydrolysis or conjugation; and (iii) several other biochemical processes, such as the induction of stress proteins or metallothioneins involved in direct interactions with the toxins and maintenance of intracellular homoeostasis **(Barker, 2002).** Under stress conditions, the snails need more energy to detoxify the toxicants and to overcome the induced stress. This may explain the high oxygen demand to comply the excessive need for energy.

On conclusion the both examined botanical materials, Thymol and Nicotine, are effective in killing the agricultural pest *E. vermiculata* and the sublethal doses of them cause adverse effects on the snail's respiration, either by inhibition or stimulation, that reflect interruption of the snail's internal physiology but further studies are needed to evaluate the efficacy of these materials as safe and economic molluscicides in the field.

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تأثير جرعات تحت مميتة لبعض مبيدات الرخويات ذات الاصل النباتى على معدل استهلاك الاكسجين فى قوقع الحدائق البنى أيوبانيا فيرميكيولاتا

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تم اجراء اختبارات معملية لتقييم فعالية بعض المواد النباتية (النيكوتين، الثيمول، المنتول، الكافيين، الكافور) كمبيدات ضد قوقع الحدائق البنى أيوبانيا فيرميكيولاتا وذلك باستخدام طريقة التطبيق الموضعى.

و أثبتت النتائج أن مادتى النيكوتين والثيمول هما الاكثر فعالية كمبيدات رخويات، حيث كانت قيمة الجرعة نصف المميتة (LD_{50}) للنيكوتين 204.02ميكروجرام/قوقع؛ بينما سجل الثيمول قيمة قدرها 551.20 ميكروجرام/قوقع. تم تحضير جرعات تحت مميتة (LD_{50}, LD_{25}) من المواد الاكثر فاعلية (النيكوتين والثيمول) ودراسة تاثيرها على معدل استهلاك الاكسجين فى القوقع أيوبانيا فيرميكيولاتا وذلك عقب فترات زمنية مختلفة من المعاملة (1، 7، 15 يوم).

اوضحت النتائج انه فقط القواقع المعاملة بالـ (LD_{50}) للثيمول اظهرت انخفاضا ذو دلالة احصائية فى معدل استهلاك الاكسجين بينما جميع المعاملات الاخرى ادت إلى زيادة معنوية فى استهلاك الاكسجين على مدار الثلاث فترات الزمنية من التقييم، وكانت هذه الزيادة اعلى ما يمكن فى حالة الجرعة المنخفضة للمادتين المختبرتين، وقد لوحظ أن مرور الزمن عقب المعاملة لم يمكن القواقع التى تم معاملتها من التخلص من التاثيرات السلبية للمواد المدروسة.

إعتماداً على نتائج الدراسة الحالية يمكن استنتاج أن المادتين المختبرتين (النيكوتين والثيمول) ذات فعالية فى قتل الافة الزراعية أيوبانيا فيرميكيولاتا؛ إلا أن تطبيق هاتين المادتين فى الحقل كمبيدات امنة واقتصادية فى حاجة الى المزيد من الدراسات .