J. Agric. Sci. Mansoura Univ., 32 (12): 10519 - 10527, 2007

Calotropis procera **GLYCOSIDES ARE MORE EFFECTIVE ON *Eobania vermiculata* (MÜLLER) THAN METHOMYL AND OTHER PLANT GLYCOSIDES.**

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ABSTRACT

Methomyl and the cardiac glycoside extract, isolated from *Calotropis procera*, were tested against the terrestrial helicid snail *Eobania vermiculata* (Müller) by the contact method. The LD_{50} values of tested materials were 153.51 and 13.87 µg/ gm of body weight respectively, which means that the extract is 11 folds more toxic to this snail than methomyl. The spectrophotototometric analysis of the cardenolide content of the tested extract proved that it is equivalent to 95 % ouabain, which is a good indication on the purity of the isolated group. At sub lethal doses of the tested extract, changes in alanine transaminases (ALT) level were higher than changes in aspartate transaminases (AST) levels. The results of this work proved that *Calotropis procera* is an important source for new and strong molluscicidal compounds that could be exploited against *Eobania vermiculata* and other species of harmful land snails.

Keywords: Calotropis procera, Eobania vermiculata, cardenolide glycocides, methomyl, molluscicidal activity, AST, ALT.

INTRODUCTON

Land mollusks are important pests of a wide range of agricultural and horticultural crops in temperate and humid habitats world-wide (Godan, 1983). Economic damage caused by snails is due to feeding and to contamination with their bodies, faeces or slime, leading to deterioration of the product quality, in addition to the financial loss (Iglesias *et al*., 2003). In Egypt, terrestrial snails attack vegetables, field crops, orchard trees as well as ornamental and medical plants (Bishara *et al*., 1968; El-Okda, 1980;; El-Wakil *et al*., 2000). The brown snail *Eobania vermiculata* (Müller) was surveyed as an agricultural economic pest in Egypt since the mid sixties of last century (Kassab and Daoud, 1964; El-Okda, 1979; Abo Bakr, 1997; Eshra 2004). Moreover, *E. vermiculata* was recorded as an intermediate host of animal nematode *Angiostrongylus cantonensis* (Aly and Sleem, 2000). Chemical control with high concentrations of pesticides, mainly metaldehyde or certain carbamates, is the main method of snail control (El-Okda, 1984; Coupland, 1996; Abdallah *et al*., 1992, 1998a). Many environmental problems such as the harmful effects against non-target organisms including mammals, poultry and wildlife result from using synthetic compounds. Some attempts were carried out to evaluate alternative, effective natural pesticides to replace the conventional synthetic pesticides (Abdelgaleil, 2005; Khidr *et al.*, 2006; El-Zemity and Radwan, 2001; Hussein *et al.* 1994, 1999, 2007a, b). The natural cardiac glycoside, ouabain, and the cardiac glycoside extract, recently isolated from *Nerium oleander,* were very toxic against the terrestrial helicid snail *Theba pisana*; however they did not exhibit molluscicidal activity against

E. vermiculata (Hussein, 2007a; Hussein *et al*., 2007a). It was clear that *E. vermiculata* is tolerant to most of the active natural molluscicidal compounds or extracts that were very effective against *T. pisana*. The cardiac glycoside extract isolated from *C. procera* was very active against *T. pisana* and *Helicella vestalis*, and was found to be tens of times more toxic to *T. pisana* and *Helicella vestalis* than methomyl (Hussien and El-Wakil, 1996). Therefore, we decided to isolate the cardenolide extract of *C. procera* and test its efficacy against *E. vermiculata* in a try to find an effective natural molluscicide that could be used to control this tolerant species. This work is a continuation of our successful attempts to find effective natural molluscicides against different species of land snails. (Hussein, 2005, 2007a, b; Hussein and El-Wakil, 1996; Hussein *et al.* 1994, 1999, 2007a, b).

MATERIALS AND METHODS

1- Snails

Adult terrestrial brown snails *Eobania vermiculata* (Müller) (family: Helicidae) were collected from pesticide-free garden in Noubaria. Snails were reared for an enough period to be fully acclimatized to laboratory conditions prior to the test.

2- Calotropis procera extract

The cardenolide extract was isolated from the latex of *Calotropis procera* according to the method described by Al-Rajhi *et al.* (2000). In brief, latex of *C. procera* was mixed with ethanol and the mixture was filtrated on Buchner funnel, the filtrate was concentrated and treated with lead acetate (50%). After filtration, the clear solution was extracted with chloroform. The chloroform layer was washed with distilled water and dried with anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to give a crystallized yellow material, which gave positive tests with reagents specific for cardenolides, Kedde and Raymond reagents.

3- Spectrophotometric determination of the cardenolide content

a- 3, 5-dinitrobenzoic acid solution

Tow grams of 3, 5-dinitrobenzoic acid was dissolved in 100 ml ethanol.

b- Sodium hydroxide solution

20 gm sodium hydroxide pellets was dissolved in distilled water and volume was made to 100 ml.

c- Ouabain and test extract standard solutions

Ouabain (95%) was purchased from Sigma, a stock solution (1000 ppm) was prepared in ethanol.1.9, 1.95, and 1.975 ml ethanol was added to 15 ml test tubes; 100, 50 and 25 µl of the stock solution were added to get the final concentrations 50, 25, 12.5 ppm, respectively; after that, 1 ml of 3, 5-dinitrobenzoic acid solution was added and tubes were mixed well with vortex, 0.1 ml of sodium hydroxide solution was added to each test tube and mixed well. The resulting color was read at 565 nm to give optical densities proportional to test concentrations.

Similar standard solutions of *C. procera* extract were prepared at the same way.

4- Bioassay

Stock solutions of tested extract and methomyl were prepared in ethanol; Tween 80® was added to the solvent (0.05 %) to prevent precipitation. Concentrated stocks were diluted with water to obtain the lower doses. Control snails were treated with the solvent. Three replicates were used for each dose, with 8 snails each. Tested dose was gently applied on the surface of the snail's mantle collar using a micropipette as the method described by Hussein *et al.,* (1994). Snails were fed on lettuce *ad libitum.* Dead snails were detected 24 hr after treatment by loss of response to a thin stainless steel needle (WHO, 1965). Toxicity values were determined by probit-analysis (Finney, 1971).

5- Sub-lethal treatments

Snails were treated with1/5, 1/10 and 1/20 of LD_{50} values of tested materials. After 24 hr of treatment, snails' tissues were prepared for biochemical tests.

6- Sample preparation for biochemical tests

Shells of snails were removed and tissues were homogenized in 10 folds (w/v) of distilled water by using glass homogenizer. Homogenates were centrifuged at 5000 rpm for 30 minutes using a cooling centrifuge at 4 °C. The supernatant was used as a source of enzyme assay.

7- Determination of protein content

Estimation of protein concentration has been carried out according to the method of Lowery *et al*. (1951) using bovine serum albumin as standard and absorbance was measured at 750 nm.

8- Aspartate transaminase (AST) and alanin transaminase (ALT) assay

In vivo effects of molluscicidal active compound(s) against AST and ALT were studied according to the method described by Reitman and Frankel (1957) using Diamond Diagnostics kit. Absorbance was measured at 546 nm.

RESULTS AND DISCUSSION

Spectrophotometric analysis of the cardenolide extract

Table 1 shows the results of the spectrophotometric analysis of the isolated extract at 565 nm by the Kedde reagent method. The extract showed almost the same optical densities at tested concentrations as those of the standard cardenolide, ouabain. These results indicate that all or most components of the extract are cardenolide compounds. The reaction enabled us to determine the standard compound or the crude extracts at concentrations as low as 0.0005 % (5 ppm). This simple method enables workers in this field to follow up the success of the progress in purification steps of the cardenolide compounds from plant extracts. The two materials gave excellent linear relationship between concentration and optical density. This means that the cardenolide content of the extract is equivalent to 95 %, because ouabain concentration was 95 %.

Table1. Spectrophotometric analysis of ouabain and cardenolide extract at 565 nm.

Material	Optical densities of tested concentrations at 565 nm		
	12.5 ppm	25 ppm	50 ppm
Ouabain (95 %)	0.08	o.16	0.34
C. procera extract	0.08	0.16	0.33

Bioassay

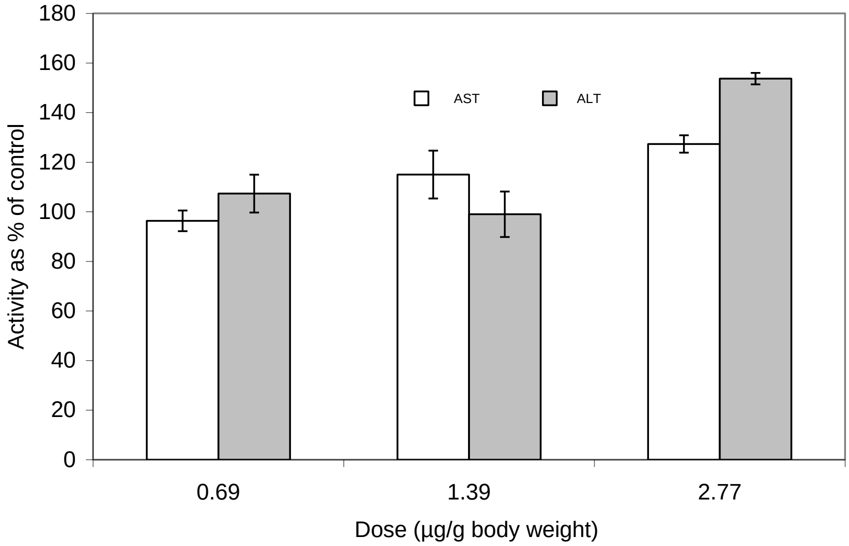
Results of contact molluscicidal activity of *C. procera* extract and methomyl after 24 hr of treatment against *E. vermiculata* are presented in Table2. The median lethal dose (LD_{50}) values of *C.procera* extract and methomyl were 13.87 and 153.51µg/g body weight respectively. It is clear that the cardenolide extract of *C. procera* is extremely toxic to *E. vermiculata* compared to the carbamate insecticide methomyl. According to their LD_{50} values, the relative potency of *C. procera* extract is 11 folds higher than methomyl. The slope values of toxicity lines of *C. procera* extract and methomyl were 6.89 and 1.61 respectively, and that means the response of tested animals to the cardenolide extract is more homogeneous than to methomyl. Although *E. vermiculata* was tolerant to the cardiac glycoside extract, isolated from *Nerium oleander*, and the natural cardiac glycoside, ouabain, which exhibited high molluscicidal activity against *T. pisana*, (Hussein, 2007a; Hussein *et al*., 2007a), it was very sensitive to the cardenolide extract of *C. procera* as proved in the present work. The molluscicidal action of the cardenolide extract was rapid, symptoms of toxicity appeared after 0.5-1 h of treatment. Snails retracted their bodies inside the shells; the ends of their feet protruded and lied on the surface of shell aperture and died in this position within 24 hr of treatment. In contrary, toxicity symptoms of methomyl appeared later, the snails' body relaxed out of the shell and became entirely paralyzed and swollen until death. This difference in toxicity symptoms indicates that the mode of action of the cardenolide extract is completely different than that of methomyl. Moreover, the big difference between slope values of toxicity lines support this belief. In the Egyptian control program of *E. vermiculata* and other land mollusks there is no use of specific molluscicide. High concentration of the carbamate insecticide methomyl (2% a.i) in wheat bran bait is the main chemical control method of terrestrial snails and slugs (Ministry of Agriculture and Land Reclamation, 2001), which presents bad adverse effects to non-target organisms of mammals, birds and honey-bees (IPCS, 1996). So, we must replace such pesticides in mollusks control programs by more specific compounds at low concentrations, in order to avoid the adverse effects on non-target organisms and to help in biodiversity conservation and environment protection.

Table2. Toxicity values of *C. procera* extract and mathomyl against *Eobania vermiculata.*

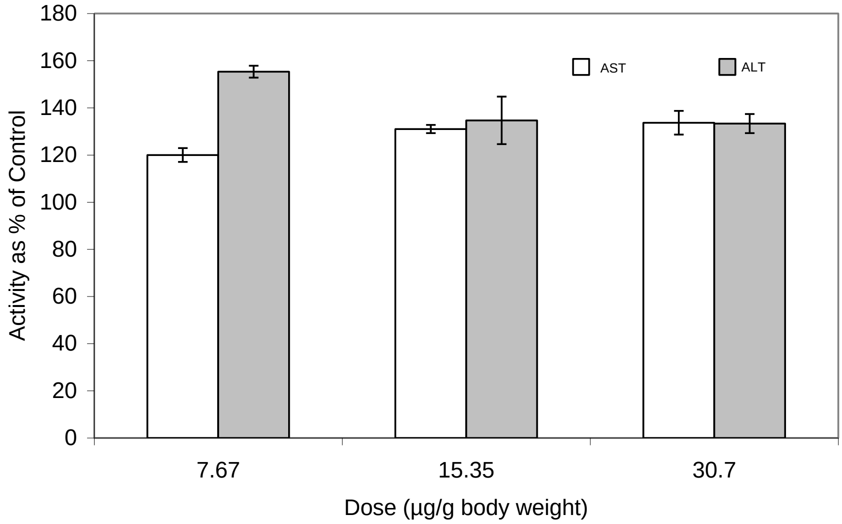
Mortality (%)	Lethal Dose (µg/g b.w)	
	***C.procera* extract**	**Methomyl**
25	11.07	58.58
50	13.87	153.51
75	17.37	402.31
90	21.28	957.55
99	30.18	4258.13
Slope	6.89 ± 0.734	1.61 ± 0.216

Biochemical effects

Transaminases (aminotransferases) constitute a group of enzymes that catalyze the interconversion of amino acid in α- keto acid by transferring amino group (Moss and Henderson, 1998). Aspartate transaminase (AST) and alanin transaminase (ALT) are located in molluscan digestive gland (hepatopancreas) and leak out into general circulation when hepatic cells are injured. The transaminases are good indicators of tissue lesion. Results of *in vivo* effect of sub-lethal doses of *C. procera* extract and methomyl on the activity of these enzymes in *E. vermiculata* are presented in Figures 1 and 2. Little increase (27 and 15 %) in AST activity was observed due to 1/5 and 1/10 the LD_{50} treatments of *C. procera* extract, respectively; however, at 1/5 LD_{50} treatment, it caused a marked increase (53%) in ALT activity (Figure 1). Sensitivity of ALT was higher than that of AST at the high dose of the cardenolide extract as shown in Figure (1), this result is similar to those obtained by Hussein *et al.* (2007a) who reported that ALT was more sensitive than AST to ouabain treatments. Methomyl treatments caused moderate and gradual increase in AST activity, at 1/20, 1/10 and 1/5 LD_{50} treatments, AST activity increased by 20, 31 and 34 % respectively (Figure 2). All methomyl treatments caused an increase in ALT activity; however the maximum increase (55 %) was due to the lower dose (1/20 of LD_{50}). Increase in AST and ALT activities in land snails treated with molluscicidal compounds was reported by many workers (El-Wakil and Radwan (1991) and Abdallah *et al*. (1998b). The results of this work proved that *Calotropis procera* is an important source for new and strong molluscicidal compounds that could be exploited against *Eobania vermiculata* and other species of harmful land snails.



Fig(1): Effect of *Calotropis procera* extract on *Eobania vermiculata* AST and ALT activities.



Fig(2): Effect of methomyl on *Eobania vermiculata* AST and ALT activities.

REFERENCES

Abdallah, E. A. M., F.A. Kassem and E. A. Kadous (1992). Laboratory and field evaluation of local bait formulations of certain pesticides against mollusca species. J. Pest Cont. Environ. Sci., 4 (2): 179-192.

Abdallah, E. A. M., F. A. Kassem, H. B. El-Wakil and Y. Abo Bakr (1998a). Molluscicidal potentiality of several pesticides against *Eobania Vermiculata* and *Theba pisana* terrestrial snails . Ann. Agric. Sci. sp. Issue, 1:263-276.

Abdallah, E. A. M., H. B. El-Wakil, F. A. Kassem, E. I. El-Agamy and Y. Abo Bakr (1998b). Impact of aldicarb and metaldehyde exposure on different molluscan enzyme activities and stress protein response. 7th Conf. Agric. Ain Shams Univ. Cairo, Dec.15-17. Ann. Agric Sci., 3: 1089 -1102.

Abdelgaleil, S. A. M. (2005). Molluscicidal and insecticidal properties of sesquiterpene lactones and extracts of *Magnolia grandiflora* L. J. Pest Cont. Environ. Sci.13(1):118.

Abo Bakr, Y. (1997). Toxicological and environmental studies on some terrestrial gastropods. M. Sc. Thesis, Faculty of Agric., Alex. Univ., Egypt.

Al-Rajhi, D., H. I. Hussein, M. S. Al-Osta and A. G. Ali (2000). Larvicidal and ovipositional activity of *Calotropis procera,* neemazal/T and *Eucalyptus* spp. against *Culex pipiens*. J. Pest Cont Environ. Sci. 8:15-26.

Aly, R. H. and S. H. Sleem (2000). Ultra structure studies on the nervous system of the land snail *Eobania vermiculata* (Gastropoda: Stylommatophora). J. Egypt. Soc. Parasitol. 30(1): 197-209.

Bishara, S. I., M. S. Hassan and A. S. Klliny (1968). Studies on some land snails injurious to agriculture in UAR. Rev. Zool. Bot. Afr.LXXVII, (3-4):239-259.

Coupland, J. B. (1996). The efficacy of metaldehyde formulations against helicid snails: the effect of concentration, formulation and species. BCPC Symp. Proc., 66: 65-72.

El-Okda, M. M. K. (1979). Land snails of economic importance at Alexandria region with some notes on the morphological features, classification, economic damage and population on the ornamental plants. Agric. Res. Rev. 57(1): 125-131.

El-Okda, M. M. K. (1980). Land snails of economic importance on vegetable crops at Alexandria and neighboring regions. Agric. Res. Rev., 58 (1): 79 – 86.

El-Okda, M. M. K. (1984). Land mollusca infestation and chemical control in El-Ismailia governorate. Agric. Res. Rev., 62 (1): 87-92.

Eshra, E. H. (2004). Studies on terrestrial mollusks at some governorates of West Delta with special reference to its integrated management. Ph.D. Thesis, Faculty of Agric., Al-Azhar Univ., Egypt.

El-Wakil, H. B and M. A. Radwan (1991). Biochemical studies on the terrestrial snail *Eobania vermiculata* (Müller) treated with some pesticides. J. Environ. Sci. Health. B26 (5&6): 479-489.

El–Wakil, H. B., F. A. Kassem, E. A. M. Abdallah and Y. Abo Bakr (2000). Ecological and biological studies on some terrestrial gastropod species in Alexandria and El- Beheira, Egypt. Alex. J. Agric. Res. 45: 207- 224.

El-Zemity, S. R. and M. A. Radwan (2001). Molluscicidal and antifeedant activity of some essential oils and their major chemical constituents against *Theba pisana* snails. Arab Univ. J. Agric. Sci. Ain Shams Univ. Cairo, 9(1):483 - 493.

Finney, D. J. (1971). Probit analysis. 3rd Ed. Cambridge Univ.Press, London, pp.318.

Godan, D. (1983). Pest slugs and snails, biology and control. Springer Verlag. Berlin pp.445.

Hussein, H. I. (2007a). Discovery of a highly active molluscicidal extract against land snails from *Nerium oleander* L. J. Agric. Sci. Mansoura Univ., 39(9): Accepted.

Hussein, H. I. (2007b). Molluscicidal activity of three plant oils against *Theba pisana* (Muller). J. Agric. Res. Kafrelsheisk Univ. Accepted.

Hussein, H. I. (2005). Composition of essential oils isolated from three plant species and their molluscicidal activity against *Theba pisana* snails. J. Pest Cont. Environ. Sci., 13(2): 15-24.

Hussein, H. I. and H. B. El-Wakil (1996). A pioneer molluscicidal and antifeeding agent from *Calotropis procera* extract, against land snails. J. Pestic. Manag. Environ. 1: 110-116.

Hussein, H. I., A. Kamel, M. Abou-Zeid, A. H. El-Sebae and M. A. Saleh (1994). Uscharin, the most potent molluscicidal compound tested against land snails. J. Chem. Ecol., 20:135-140.

Hussein, H. I., D. Al-Rajhy, F. El-Shahawi and S. Hashem (1999). Molluscicidal activity of *Pergularia tomentosa* (L), methomyl and methiocarb against land snails. Int. J. Pest. Manag. 45: 211-213.

Hussein, H. I., E. H. Eshra and Y. Abo Bakr (2007b). Molluscicidal activity and Biochemical effects of certain monoterpenoids against land snails. J. Adv. Agric. Res., 12(4): Accepted.

Hussein, H. I., Y. Abo Bakr and E. H. Eshra (2007a). Molluscicidal activity and biochemical effects of two phyto-glycosides against land snails. J. Adv. Agric. Res., 12(4): Accepted.

Iglesias, J., J. Castillejo and R. Castro (2003). The effects of repeated applications of the molluscicide metaldehyde and the biocontral nematode *Phasmarhabditis hermaphrodita* on mollusks, earth worms, nematodes, acarids and collembolans: a two-years study in north–west Spain. Pest Manag. Sci., 59: 1217-1224.

Khidr, F. K., W. M. Gabr, A. S. Yousif and S. S. Hussein (2006). Biochemical effects of two natural pesticides on the brown garden snail *Eobania vermiculata* Muller. Egypt. J. Agric. Res., 84(3):713-

Kassab, A. and H. Daoud (1964). Notes on biology and control of land snails of economic importance in the U. A. R. Agric. Res. Rev., 42: 77-98.

Lowery. O. M., N. J. Rosebrough, A. L. Farr and R. J. Randoll (1951). Protein measurement with follin phenol reagent. J. Biol. Chem. 193: 256-257.

Ministry of Agriculture and Land Reclamation (2001). Technical recommendations for agricultural pests control. pp. 180-184.

Moss, D. W. and A. R. Henderson (1998). Enzymas. In: Burtis, C. A. and E.R. Ashwood (eds), Tietz Fundamentos de Quimica Clinica, 4th ed., Guanbara Koogan Rio de Janeiro, pp 275-325.

Reitman, S. and S. Frankel (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic trnsaminase. Am. J. Clin. Path., 28:56-63.

IPCS (1996). Environmental Health Criteria 178: Methomyl. Geneva, World Health Organization.

WHO (1965). Molluscicidal screening and evaluation. Bull., 38: 507-581.

جليكوسيدات كالوتروبيس بروسيرا أكثر فاعلية على قوقع ايوبانيا فيرميكيولاتا من الميثوميل والجليكوسيدات النباتية الأخرى

ياسر أبو بكر ، السيد حسن عشرة و حمدى ابراهيم حسين
معهد بحوث وقاية النباتات- الصبحية- الاسكندرية

أختبرت الكفاءة الابادية لمجموعة الجليكوسيدات المستخلصة من نبات كالوتروبيس بروسيرا (العشار) ضد القوقع الأرضى البنى، ايوبانيا فيرميكيولاتا، مقارنة بمبيد الميثوميل وذلك بطريقة الملامسة. وأظهرت النتائج ان المستخلص الجيلكوسيدى شديد السمية ضد القوقع المختبر حيث كانت الجرعة القاتلة النصفية 87و13 ميكروجرام /جرام من وزن الجسم بعد 24 ساعة من المعاملة وذلك يعادل أكثر من 11 ضعف سمية مبيد الميثوميل لنفس القوقع، حيث كانت الجرعة القاتلة النصفية 51و153 ميكروجرام /جرام من وزن الجسم بعد نفس الزمن من المعاملة. وأوضحت نتائج التحليل اللونى أن طيف امتصاص تركيزات مختلفة من المستخلص تكافىء طيف امتصاص نفس التركيزات للجليكوسيد القياسى أوابين (تركيز 95 %) مما يدل على مدى نقاوة المجموعة المستخلصة من هذا النبات. الجرعات المنخفضة غير المميتة للمستخلص أحدثت زيادة فى نشاط انزيم الانين ترانس أمينيز مقارنة بتأثيرها على انزيم اسبارتيت ترانس أمينيز، بينما كان تأثير مبيد الميثوميل على كل من الانزيمين أوضح من تأثير المستخلص عليهما.