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# LARVICIDAL AND OVIPOSITIONAL ACTIVITY OF *CALOTROPIS PROCERA*, NEEMAZAL/T *AND EUCALYPTUS* SPP. AGAINST *CULEX PIPIENS*

By

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## ABSTRACT

NeemAzal/T, a commercial formulation containing 5 % azadirachtin (AZ), a cardenolide extract isolated from *Calotropis procera* R.Br. (Asclepiadaceae), and a *Calotropis procera* extract purchased from Sigma were tested for their larvicidal activity against first and fourth instar larvae of *Culex pipiens* at 23 ± 2 °C. The $LC_{50}$ values for the three materials against the 1st instar larvae, after 96 h, were 0.74, 4.8, and 4.1 mg /l, respectively. $LT_{50}$ values for AZ against the fourth instar larvae were 4.6, 3.4, 3.25, and 3.1 days at 5, 7.5, 10, and 12.5 mg/l, respectively. The $LC_{50}$ value for the isolated extract against the 4 th instar larvae was 27 mg/l, after 72 h. The isolated extract and the volatile oils of *Eucalyptus camaldeulensis* Dehnh. and *E. fideroxylon* (Myrtaceae) inhibited oviposition at 50 mg/l, whereas AZ inhibited oviposition at 2.5 mg/l.

*Keywords*: Azadirachtin, *Calotropis procera, Culex pipiens, Eucalyptus,* larvicides, mosquito, neem, oviposition.

## INTRODUCTION

Natural products are well known to have a range of useful biological properties. Many of the earliest insecticides were extracts of plants (Casida, 1973; Jacobson and Crosby, 1971; Ware, 1980). Much

interest has developed using plant extracts as insecticides for mosquito control, to overcome the problems of conventional pesticides (Chou *et al.*, 1997; Thangam and Kathiresan, 1997; Murugan *et al.*, 1996; Perich *et al*, 1994; Sinniah *et al.*, 1994; Collins *et al.*, 1993; Rao *et al.*, 1988). Extracts from *Calotropis* spp. were shown to have insecticidal properties against mosquito (Kalyanasundaram and Das, 1985; Saxena and Sumithra, 1986; Girdhar *et al.*, 1984). Recently, we have found that cardiac glycosides from *Calotropis procera* R.Br. and *Pergularia tomentosa* L. are very promising molluscicides against harmful land snails, and may play an important role in this field (Hussein *et al.*, 1994; Hussein and El- wakil, 1996; Hussein *et al.*, 1999). In this work, we report on the insecticidal properties of a cardenolide (cardiac glycoside) extract from *Calotropis procera* against *Culex pipiens,* compared to those of NeemAzal/T and *Eucalyptus* spp.

## MATERIALS AND METHODS

### Test Extracts

### The cardenolide extract

The Latex of *C. procera* was collected in amber glasses. The crude cardenolide extract was isolated from the latex according to the method of Hesse and Reicheneder (1936) with some modifications. We did not use alkali to neutralize the final chloroform extract; instead, we washed it several times with distilled water. We also evaporated the chloroform extract to dryness to get the cardenolide extract instead off precipitating it with petroleum ether. TLC using three solvent systems: chloroform: methanol (9: 1), ethyl acetate – methanol (84- 16), and ethyl acetate-methanol (97:3) showed several spots and was similar to that of *C. procera* extract purchased from Sigma. However, the major compound in our extract (with $R_f$ values of o.25, o.55, and 0.35 in the three solvent systems respectively) was absent in Sigma extract. The two extracts showed the same reactions to Baljet, Kedde, and Raymond reagents. The major compound in our extract gave purple color with Kedde reagent and blue color with $H_2SO_4$ . After exposure to UV and air, it took the yellow color on the plate. Our extract was yellow, crystalline, with strong unpleasant odor, while the Sigma extract was white powder,

without any characteristic odor. Thin layer chromatography was carried out using 0.25 mm silica gel 60 $F_{254}$ plates (5x10 cm) from Merck Company.

**Volatile oils of *Eucalyptus* SPP.**

About 300 g leaves of *Eucalyptus camaldulensis* Dehnh. and *E. fideroxylon* (Myrtaceae), collected in September from trees around the faculty of Agriculture, in Riyadh, was macerated with hexane on room temperature. After 7 d, the hexane extract was filtered and evaporated under reduced pressure to give an oily residue. The oily residue was steam distilled to give a distillate and residue. The distillate was extracted with diethyl ether. The ether layer was dried using anhyd. sodium sulfate. Ether was evaporated under reduced pressure to give the yellowish oil with its Characteristic odor. Identification of the plants was carried out by the Botany Department, King Saud University.

**Neem Extract**

Trifolio-M, Germany, NeemAzal/T (5 % azadirachtin) was kindly provided by Prof. Dr. Ibrahim Kilany, Department of Zoology, College of Science, King Saud University. Concentrations used in this work are calculated as azadirachtin (AZ).

**Preparation of test solutions**

A known weight of *C. procera* extracts was dissolved in the minimum volume of 95 % ethanol (containing 1% Tween 80) and diluted with distilled water to obtain the highest concentrations, dimethylsulfoxide was added before dilution to help dissolution of Sigma extract. Lower concentrations were prepared by further dilution with distilled water. The highest concentration of ethanol was 0.06 %. In case of first instar larvae tests, the highest conc. of Tween 80 and dimethylsufoxide in the final test solution were 0.00055 % and 0.011 %, respectively. Concentrations of *Eucalyptus* volatile oils and AZ were prepared by dissolving the material in the minimum volume of ethanol and Tween 80 only, and dilution with distilled water. Controls were prepared using the highest concentrations of same solvents and emulsifier.

**Bioassays**

The bioassays were conducted on strain of *C. pipiens*, originally collected in Riyadh. Larvae were reared at 25 $\pm$ 2 °C and fed finely

screened mouse food. Adults were maintained at the same temperature and fed 10 % sucrose solution. Females were blood fed on a pigeon to get egg masses.

**Larvicidal activity**

Twenty 4 th or 1st instar larvae of the mosquito, *C. pipiens* were placed into 100 ml glass beakers (5.4 cm diam. x 6.7 cm height) containing 50 ml of control or test solutions. Ikeda *et al.* (1998) used glass vials ( 2.5 cm diam., 4.5 cm height ) containing 5 ml of test plant extracts against the fourth instar larvae of *C. quinquefasciatus* ( each vial contained 15 larvae ). Larvae that did not move when touched with a needle were considered as dead . Three replicates were used at each concentration. Tests were carried out at 25 $\pm$ 2 °C. Preliminary tests were carried out to get the appropriate range of tested concentrations. Probit analysis of results was carried out according to Finney (1971).

**Effect on mosquito oviposition**

Many bioassay methods have been used to evaluate compounds and extracts for effects on specific behavioral steps associated with mosquito oviposition (Benzon and Apperson 1988; O,Gower, 1963; Pile *et al.* 1991). In this study, we used the simple egg-raft counting bioassay method. Treatments and control cups (100 ml each) were introduced into bioassay cages (45x 45 x 45 cm) containing gravid mosquitoes; the cups were distributed randomly in the cages. Egg rafts laid in each cup, up to three days, were tabulated.

## RESULTS AND DISCUSSION

**Effect of plant extracts on first instar larvae**

Results of larvicidal activity of tested plant extracts against the 1st instar larvae of *C. pipiens* are presented in Table 1. It can be seen that there is no big difference between the activity of the isolated cardenolide extract and the activity of Sigma extract. The $LC_{50}$ values for the two extracts were 4.8 and 4.1 mg/l, respectively ( Table 2 ). At 6 mg/l, the two extracts caused 73.3 % mortality after 96 h. After 8 days, this concentration caused 88.3 % mortality. Larvae treated with this and with higher concentrations did not develop to higher stages and died after

several days. This effect may be attributed to the antifeeding activity of the cardenolides in this extract and/or to its insect growth regulating activity, since these cardenolides have a steroidal structure. Cardenolides are highly toxic and antifeeding to birds and to land snails (Prower and Prower,1968; Hussein and El-Wakil, 1996; Hussein *et al.*, 1994; Hussein *et al.*, 1999 ).

**Table (1) :. Larvicidal activity of the cardenolide extract (isolated), Sigma extract, and azadirachtin against 1 st-instar larvae of *C. pipiens* at 23 ± 2 °C.**

| The isolated extract* | | Sigma extract | | Azadirachtin | |
|---|---|---|---|---|---|
| Conc. (ppm) | % (M)** | Conc. (ppm) | % (M) | Conc. (ppm) | % (M) |
| 10 | 98.3 a | 10 | 93.3 a | 1.50 | 90 a |
| 8 | 73.3 b | 8 | 83.3 b | 1.00 | 61.7 a |
| 6 | 73.3 b | 6 | 73.3 b | 0.50 | 23.3 b |
| 4 | 25 c | 4 | 38.3 c | 0.25 | 8.3 b |
| 2 | 10 d | 2 | 20 d | - | - |
| 0.0 | 0.0 e | 0.0 | 0.0 e | 0.00 | 0.0 b |

* Original data were transformed into arcsin percentage before ANOVA and Fisher's protected LSD tests.

** Values followed by the same letter within a column are not significantly different at the 0.05 level .

% (M) = Average % mortality.

The $LC_{50}$ value for AZ after 96 h, was 0.74 mg/l (Table 2). At 0.25 and 0.5 mg/l, aza caused 8.3 and 23.3 % mortality, respectively. However, these two concentrations caused 66.7 and 90 % mortality after 8 days. Neem extracts have no direct toxicity, but inhibits development of mosquito larvae. Mosquito larvae treated with neem extracts died several days after treatment ( Schmutterer and Zebitz, 1984 and Murugan *et al.*, 1996).

**Table (2) :. Probit analysis for larvicidal activity of the cardenolide extract (isolated), the cardenolide extract (Sigma) and azadirachtin against 1 st instar larvae of *C. pipiens* after 96 h, at 23 ± 2 °C.**

| Material | $LC_{50}$ (ppm) | 95 % fiducial limits | $LC_{95}$ (ppm) | 95 % fiducial limits | Slope ± SE |
|---|---|---|---|---|---|
| Isolated extract | 4.8 | 4.35-5.27 | 11.78 | 9.87-14.12 | 4.21 ± 0.18 |
| Sigma extract | 4.1 | 3.6-4.59 | 12.63 | 10.1-15.93 | 3.34 ± 0.13 |
| Azadirachtin | 0.74 | 0.65-0.84 | 2.23 | 1.71-2.92 | 3.43 ± 0.14 |

**Effect of plant extracts on 4th instar larvae**

Symptoms of toxicity of the isolated cardenolide extract began two hours after treatment. Larvae treated with this extract tended to float on the water surface. They did not sink to the bottom for more than five seconds every several minutes. After 24 h they did not respond to external stimuli, such as putting hand above the beaker, knocking on the beaker wall, or blowing air on the water surface Control larvae sank quickly to the bottom as soon as exposed to any of these stimuli. Death occurred after 48 h with convulsions and tremors. Most of dead larvae had curved bodies. The $LC_{50}$ value of this extract was 27 mg/l after 72 h. Kalyanasaundaram and Das ( 1985 ) isolated a petroleum ether extract from *Calotropis* sp and tested it against 4 th-instar larvae of *C. quinquefasciatus* ( formerly *C. pipiens* ). The $LC_{50}$ of their extract was 90.1 mg/l. Girdhar *et al.* (1984) found that the latex of *C. procera* was toxic to the larvae and eggs of three mosquito spp. at 10000 mg/l. The high concentrations used by these workers, compared to ours, prove that the cardenolides are the active principles against mosquito larvae.

On the other hand, larvae treated with AZ showed normal behavior and responded to external stimuli up to two days after treatment. The $LT_{50}$ values for AZ decreased with the increase in

concentrations (Table 3). At 5 and 12.5 mg/l, $LT_{50}$ values for AZ were 4.6 and 3.1 days, respectively. Generally, these results agree with those of Schmutterer and Zebitz (1984) and Singh (1984).

**Table (3) :. Larvicidal activity of azadirachtin against 4 th instar larvae of *C. pipiens*, at 23 ± 2 °C.**

| Conc. (ppm) | Mortality % after day | | | | | $LT_{50}$ (days) |
|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| 5 | 5 | 41.7 | 61.6 | 73.3 | 91.7 | 4.6 |
| 7.5 | 31.7 | 75 | 83.3 | 88.3 | 91.7 | 3.4 |
| 10 | 38.3 | 78.3 | 86.7 | 93.3 | 100 | 3.25 |
| 12.5 | 46.7 | 76.7 | 88.3 | 90 | 100 | 3.1 |

**Table (4):. Number of egg rafts laid by gravid *C. pipiens* in water containing different concentrations of tested plant extracts.**

| Conc. (mg/l) | *C. procera* extract | Volatile oils of *Eucalyptus sp.* | Conc. (mg/l) | Azadirachtin |
|---|---|---|---|---|
| 0.0 | 4 | 2 | 0.0 | 4 |
| 5 | 2 | 1 | 0.25 | 5 |
| 10 | 3 | 1 | 0.5 | 8 |
| 20 | 2 | 8 | 1 | 2 |
| 50 | 0.0 | 0.0 | 2.5 | 0.0 |

**Ovipositional activity**

Results of the ovipositional activity of the three tested plant extracts are recorded in Table 4. We have repeated this test several times. Only the highest concentration of each extract inhibited oviposition. Different numbers of egg rafts were laid in water containing the lower concentrations. There was no correlation between the concentration and the number of egg rafts laid by gravid females. Leave extracts of *Calotropis gigantia*, at 100 mg/l, inhibited mosquito oviposition (Saxena and Sumithra, 1986). Neem extracts at the concentrations of 2.5, 5, 7.5, 10, and 20 mg/l did not affect the oviposition behavior of *Culex quinquefasciatus*, but, egg rafts placed in these concentrations had significantly reduced hatch rates (Zebitz, 1987).

At 2.5 mg/l, AZ inhibited oviposition, whilst at lower concentrations (0.25, .5, and 1 mg/l), it had strong detrimental effects on new hatch larvae, they did not develop normally and died after several days. Also, new larvae hatched in the concentrations of 5, 10, and 20 mg/l of the cardenolide extract did not develop to the higher stages and died after several days. Mortality rates at the concentrations of 5, 10, and 20 mg/l were 79, 100,and 100%, respectively after 7 days. The volatile oils of *Eucalyptus* spp. prevented oviposition at 50 mg/l and had no effect on the new hatch larvae at the lower concentrations. The lower concentrations of both AZ and the cardenolide extract may be used as traps for mosquito egg rafts, since these concentrations are toxic to first instar larvae. The results of this work indicate the possibility of using extracts from *C. procera* in controlling mosquito populations on stagnant pools and natural ponds.

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## الملخص العربى

# التأثير الابادى لنبات العشار ومبيد نيمازال-ت ونبات الكافور على يرقات بعوض الكيولكس وتأثيرهما على وضع البيض

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تم دراسة التأثير الابادى لمبيد نيمازال -ت ( مبيد تجارى يحتوى ٥% ازادايركتين) ومستخلص كاردينوليد معزول من نبات العشار، ومستخلص لنبات العشار من شركة سيجما ضد يرقات العمر الاول والعمر الرابع لبعوض الكيولكس على درجة حرارة ٢٣± ٢°م، وكانت قيم التركيزات القاتلة ل ٥٠% من يرقات العمر الاول للمواد الثلاثة بعد ٩٦ ساعة هى ٠,٧٤، ٤,٨، ٤,١ مجم/ لتر على التوالى. وكان الزمن اللازم لقتل ٥٠% من يرقات العمر الرابع بالازادايركتين هو ٤,٦، ٣,٤، ٣,٢٥، ٣,١ يوم بتركيزات ٥، ٧,٥، ١٠، ١٢,٥ مجم/ لتر على التوالى. كان التركيز القاتلة لـ ٥٠% ليرقات العمر الرابع بالمستخلص المعزول بعد ٧٢ ساعة هو ٢٧مجم/لتر. ثبط كل من المستخلص المعزول وزيوت الكافور المتطايرة وضع البيض عند تركيز ٥٠مجم / لتر، بينما ثبط الازادايركتين وضع البيض عند تركيز ٢,٥ مجم/لتر.