Journal of Natural Products
Vol. 47, No. 4, pp. 702-705, Jul-Aug 1984

CORRELATION OF THE ANTICHOLINESTERASE AND MOLLUSCICIDAL ACTIVITY OF THE LATEX OF *EUPHORBIA ROYLEANA* ON THE SNAIL *LYMNAEA ACUMINATA*

D.K. SINGH and R.A. AGARWAL*

Department of Zoology, University of Gorakhpur, Gorakhpur 273 001, India

Synthetic organic pesticides, such as chlorinated hydrocarbons and organophosphorous and carbamate compounds, are widely used for the control of pests and vectors in spite of the fact that these pesticides are now causing serious environmental hazards (1-4). The search goes on for pesticides of plant origin because of their rapid biodegradability. However, to date, only a few pesticides of plant origin (pyrethroids, nicotinoids, and rotenoids, etc.) are available. This is a pioneer report on the molluscicidal properties of the latex of *Euphorbia royleana* Boiss, a cactus-like plant, which is extremely hardy and is abundantly found throughout India; it can be planted by sowing a piece of the stem about 5 cm in soil. Water requirement is very little, and fertilizers are not needed for its growth. It has a quick knockdown action and causes high mortality of the snail *Lymnaea acuminata*, the intermediate host of liver flukes, *Fasciola hepatica* and *Fasciola gigantica* (5). In the present paper, we report on the anti-acetylcholinesterase effect of the latex of *E. royleana* on the nerve tissue acetylcholinesterase of the snail *L. acuminata*.

MATERIAL AND METHODS

E. royleana (local name, Sehur) was collected locally and identified by the Botany Department, University of Gorakhpur, India, and a specimen, voucher number 994/83 was deposted in the Herbarium. The white latex was collected in glass beakers by cutting the stem apex and allowing the latex to drain. A 3% (v/v) solution of the latex was prepared in distilled H_2O and centrifuged at 2000 *g* for 20 min. The supernatant was lyophilized at −40°. The lyophilized powder from 3 ml latex was sequentially extracted with 5 ml each of Me_2CO, Et_2O, EtOH, CCl_4, and $CHCl_3$. Centrifugation at 2000 *g* for 20 min was carried out after each extraction, and the solvent was allowed to evaporate from each supernatant. The wet weight of 1 ml latex was 895 mg, and the dry weight, 360 mg/ml. The dry weight of the organic-solvent-extracted latex was 229 mg/ml.

The snails were treated *in vivo* for 24 h in aquaria having 3×10^{-6}, 6×10^{-6}, 9×10^{-6}, and 12×10^{-6} (v/v) latex of *E. royleana*, extracted in H_2O or extracted through a series of organic solvents. Twenty snails were kept in every aquarium with 2 liters of H_2O. The toxicity of nicolsamide (5-chlorosalicylic-2′-cholor-4′-nitroanilide, Bayer India Ltd.); eserine [5-methyl-carbamoyloxy-1, 3a, 8- trimethyl-2,3,3a, 8a-tetrahydropyrrolo (2, 3-b)-indol, Sigma Chemical Co., USA]; phorate, *O, O*- diethyl-*S* [(ethylthio) methyl] phosphorodithioate, All India Medical Corporation (30 mg/liter); formothion, *O, O*-dimethyl S (*N*-methyl-*N*-formoyl carbamolymethyl) dithiophosphate, Sandoz India Ltd.) (27 mg/liter); mexacarbate, 4-dimethylamino-3, 5-xylyl methylcarbamate, Dow Chemicals, New York (2.5 mg/liter); and carbaryl, 1-naphthyl methylcarbamate, Union Carbide India Ltd. (14 mg/liter) was also determined in similar manner and compared with latex. The doses were based on toxicity studies carried out by Singh (6). Controls were kept in H_2O alone. After 24 h, the acetylcholinesterase (AChE) activity was measured in the nerve tissue of treated and control snails. AChE activity was measured by the method of Ellman *et al.* (7) as modified by Singh and Agarwal (8) for this snail. Nerve tissue (50 mg) around the buccal mass was homogenized in 1.0 ml of 0.1 M phosphate buffer, pH 8.0, for 5 min in an ice bath and centrifuged at 1000 *g* for 30 min at −4°.

The enzyme-containing supernatant fraction (0.05 ml) was pipetted to a cuvette. To this was added 10 μl (5×10^{-4} M) of freshly prepared acetylthiocholine iodide solution in distilled H_2O, 1.45 ml of buffer (pH 8.0), and 0.05 ml of the chromogenic agent 5:5 dithio-*bis*-nitrobenzoate (DTNB). The change in optical density at 412 mμ, caused by the enzymatic reaction, was monitored for 3 min at 25°.

The *in vitro* effect of latex of *E. royleana* (5×10^{-7}, 1×10^{-6}, 1.5×10^{-6}, 2×10^{-6}) (v/v) extracted with H_2O and organic solvents, was also studied on AChE activity. The required amount of lyophilized latex was dissolved in phosphate buffer (1.45 ml) and preincubated

with the 0.05 ml enzyme at 25° for 15 min; 10 µl of substrate (5×10^{-4} M) and 0.05 ml DTNB were mixed and enzyme activity was measured at 412 mµ. In the control group, latex-free buffer was used. Organic pesticides-phorate (0.50 µg), formothion (0.75 µg), mexacarbate (1.0 µg), and carbaryl (2.0 µg) were dissolved in Me_2CO and requisite amounts pipetted into the cuvette. The Me_2CO was allowed to evaporate and then 0.05 ml of enzyme was preincubated with each of the pesticides for 15 min. Enzyme activity was measured as before. In the control experiments, Me_2CO alone was used. Protein estimation was done by the method of Lowry *et al.* (9). Enzyme activity has been expressed as µM SH hydrolyzed $min^{-1}mg^{-1}$ protein. Each experiment was replicated at least six times, and data have been expressed as mean±SE. Student's *t*-test was used for locating significant differences, while analysis of variance was used for studying the dose-dependent effect of the latex on cholinesterase inhibition.

RESULTS

Exposure to latex of *E. royleana* resulted in typical symptoms of nerve poisoning such as excitation and convulsions followed by paralysis. The symptoms became apparent within an hour of treatment, and death took place within 24 h. LC_{50} of the latex was 7.5×10^{-6} (v/v) (1.91 ppm dry weight) while that of nicolsamide, a standard molluscicides, was 11.8 ppm; treatment with 9×10^{-6} (v/v) latex caused 63% snail mortality while concentrations higher than $2.7-10^{-5}$ (v/v) caused 100% mortality within 24 h.

Table 1 shows that *in vivo* treatment with latex of *E. royleana* for 24 h caused a significant ($p<0.05$) dose-dependent inhibition of AChE activity in the nerve tissue of *L. acuminata*. Thus, a concentration of 3×10^{-6} (v/v) reduced enzyme activity to 87% while 12×10^{-6} (v/v) of latex reduced it to 50% of the control values. Latex obtained from sequential extraction with organic solvents also showed a similar inhibition of AChE activity. The anti-AChE property of sequentially extracted latex, though statistically insignificant, was lower than the water extracted latex, at all concentrations (Table 1).

In vivo treatment with the anti-AChE pesticides, phorate (30 mg/liter), formothion (27 mg/liter), mexacarbate (2.5 mg/liter), and carbaryl (14 mg/liter) reduced the enzyme activity to 67%, 79%, 53%, and 73% of the controls respectively (Table 2).

In vitro exposure to the latex of *E. royleana* significantly inhibited the AChE activity after 15 min of preincubation (Table 3). *In vitro* treatment with both water-extracted and organic solvent-extracted latex of *E. royleana* showed significant ($p<0.05$) dose-dependent inhibition of AChE activity (Table 3). The anti-AChE activity of latex extracted with organic solvents was lower in comparison to the latex extracted with H_2O (Table 3).

In vitro treatment with phorate (0.5 µg w/v), formothion (0.75 µg w/v), mexacarbate (1 µg w/v), carbaryl (2 µg

TABLE 1. The 24-h *In Vivo* Inhibition of Acetylcholinesterase from the Nerve Tissue of *Lymnaea acuminata* by the Freeze-Dried Aqueous Latex of *Euphorbia royleana*

Nature of latex	AChE activity: µm SH hydrolyzed/min/mg protein				
	Control	3×10^{-6} (v/v)[a]	6×10^{-6} (v/v)	9×10^{-6} (v/v)	12×10^{-6} (v/v)
Supernatant of aqueous solution of latex	0.070±0.0012[b] (100)[c]	0.061±0.0013 (87)	0.049±0.0007 (70)	0.040±0.0006 (57)	0.0352±0.0004 (50)
Latex Serially[d] extracted	0.072±0.0006 (100)	0.064±0.0007 (88)	0.0548±0.0006 (76)	0.044±0.0004 (61)	0.040±0.0006 (56)

[a]Dose (v/v) has been expressed as final concentration in aquarium water.

[b]Values are Mean±SE of six replicates ($p<0.01$) when *t*-test was applied to see whether significantly different from control. ($p<0.05$) when analyses of variance was applied to see whether AChE inhibition was dependent on concentration of latex.

[c]Values in parentheses indicates percent enzyme activity with controls taken as 100%.

[d]Serial extraction was carried out through Me_2CO-Et_2O-EtOH-CCl_4 and $CHCl_3$.

TABLE 2. *In Vivo* Inhibition of Acetylcholinesterase in the Nerve Tissue of *Lymnaea acuminata* by Organophosphate and Carbamate Pesticides

AChE activity: μm SH hydrolyzed/min/mg protein				
Control	Phorate (30 mg/liter)[a]	Formothion (27 mg/liter)	Maxacarbate (2.5 mg/liter)	Carbaryl (14 mg/liter)
0.075±0.0006[b] (100)[c]	0.049±0.0006 (67)	0.059±0.0008 (79)	0.048±0.0013 (53)	0.055±0.0007 (73)

[a]Doses were lower than LC_{50} for 24 h treatment.
[b]Values are Mean±SE of six replicates. ($p<0.01$) when *t*-test was applied to see whether significantly different from control.
[c]Values in parentheses indicate percent enzyme activity with controls taken as 100%.

TABLE 3. *In Vitro* Inhibition of Acetylcholinesterase of the Nerve Tissues of *Lymnaea acuminata* by the Freeze-Dried Aqueous Latex of *Euphorbia royleana*

Nature of latex	AChE activity: μm SH hydrolyzed/min/mg protein				
	Control	0.5×10^{-6} (v/v)[a]	1.0×10^{-6} (v/v)	1.5×10^{-6} (v/v)	2.0×10^{-6} (v/v)
Supernatant of aqueous solution of latex	0.075±0.0068[b] (100)[c]	0.067±0.0005 (89)	0.0585±0.0008 (78)	0.0392±0.0006 (52)	0.025±0.0006 (33)
Latex[d] Serially extracted	0.073±0.0004 (100)	0.071±0.0004 (97)	0.062±0.0003 (85)	0.042±0.0005 (58)	0.033±0.0006 (45)

[a]Concentration (v/v) of freeze-dried latex has been given as a final concentration in the incubation mixture present in the curvette.
[b]Values are Mean±SE of six replicates ($p<0.01$) when *t*-test was applied to see whether significantly different from control. ($p<0.05$) when analyses of variance was applied to see whether AChE inhibition was dependent on concentration of latex.
[c]Values in parentheses indicates percent enzyme activity with controls taken as 100%.
[d]Sequential extraction was carried out through Me_2CO-Et_2O-EtOH-CCl_4 and $CHCl_3$.

w/v), and eserine (0.23 μg/ w/v) reduced enzyme activity to 42%, 47%, 39%, 47%, and 13% of the control, respectively (Table 4).

DISCUSSION

The toxicity experiments carried out in this study have demonstrated that the latex of *E. royleana* has quick knockdown action, and its molluscicidal activity was six times higher than nicolsamide, a standard molluscicide (10-12). The low doses causing this are indicative of the presence of a very active compound in the latex.

The present study also shows that the latex of *E. royleana*, a strong molluscicide, contains a potent anti-acetylcholinesterase substance active in *in vivo* and *in vitro*. The active substance is temperature resistant and soluble both in H_2O and organic solvents. Even though the exact concentration of the active substance in the latex is not known, the

TABLE 4. *In Vitro* Inhibition of Acetylcholinesterase of the Nerve Tissue of *Lymnaea acuminata* by Organophosphorus and Carbamate Pesticide

Control	AChE activity: μm SH hydrolyzed/min/mg protein				
	Eserine (0.23 μg w/v)	Phorate (0.5 μg w/v)	Formothion (0.75 μg w/v)	Mexacarbate (1.0 μg w/v)	Carbaryl (2.0 μg w/v)
0.076±0.0004 (100)	0.0059±0.0004[a] (13)[b]	0.032±0.003 (42)	0.036±0.0003 (47)	0.030±0.0005 (39)	0.051±0.0073 (67)

[a]Values are the Mean±SE of six replicates. ($p<0.01$) where *t*-test was applied to see whether significantly different from control.
[b]Values in parentheses indicate percent enzyme activity with controls taken as 100%.

crude extract of the latex showed anti-AChE activity of the same order of magnitude as synthetic pesticides (13). The anti-AChE activity of solvent extracted latex was somewhat lower than the aqueous extracted latex; the difference may be due to the physical loss of the active compound during extraction.

The present study, though not conclusive, gives ample evidence that the mortality of snails caused by the latex of *E. royleana* may be because of its strong anti-AChE activity. To confirm this, further purification and identification of the active compound is needed.

LITERATURE CITED

1. R.F. Reidinger, *J. Wildlife Mgmt.*, **40**, 677 (1976).
2. R.S. Panwar, D. Kapoor, H.C. Joshi, and R.A. Gupta, *J. Inland Fish. Soc. India*, **8**, 129 (1976).
3. D.R. Clark, Jr., T.H. Kunz, and T.E. Kaiser, *J. Mammalolgy*, **59**, 84 (1978).
4. D.S. Mathur, *Zoologiana*, **3**, 23 (1980).
5. O. Singh and R.A. Agarwal, *J. Econ. Entomol.*, **74**, 568 (1981).
6. D.K. Singh, Ph.D. Thesis, University of Gorakhpur, India, 1982.
7. G.L. Ellman, K.D. Courtney, V. Andres, Jr., and R.M. Featherstone, *Biochem. Pharmacol.*, **7**, 88 (1961).
8. D.K. Singh and R.A. Agarwal, *Zool. Jb. Physiol.*, **86**, 315 (1982).
9. O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1952).
10. A.S. Luis, S.R. Lawrence, and H.B. Wessel, *Bull. W.H.O.*, **39**, 316 (1968).
11. H. Hoestlandt, *C.R. Acad. Sc. Paris*, **272**, 1912 (1971).
12. J.A. Reinert, *Proc. Flo Stata Hortic. Soc.*, **85**, 380 (1972).
13. D.K. Singh and R.A. Agarwal, *Comp. Physiol. Ecol.*, **7**, 191 (1982).

Received 9 May 1983