Toxic Effects of Crotocaudin Extracted from the Medicinal Plant *Croton tiglium*

Ram P. Yadav and Ajay Singh*

Department of Zoology, D. D. U. Gorakhpur University, Gorakhpur (U. P.) – 273009, India.
E-mail: singhajay_gkp@rediffmail.com

* Author for correspondence and reprint requests

Z. Naturforsch. **65c**, 327–336 (2010); received September 18, 2009/January 7, 2010

The compound crotocaudin extracted from the stem bark of the medicinal plant *Croton tiglium* Linn. was administered for 24 h or 96 h to the freshwater vector snail *Lymnaea* (Radix) *acuminata* Lamarck in order to test its toxicity. *L. acuminata* is the intermediate host of *Fasciola hepatica* and *Fasciola gigantica* which cause immense harm to man and his domestic animals. It was observed that the molluscicidal activity of crotocaudin against *L. acuminata* is time- as well as dose-dependent. There was a significant negative correlation among LC_{50} values and exposure periods, *i.e.* increasing the exposure time, the LC_{50} value of crotocaudin decreased from 5.37 μM (24 h) > 2.08 μM (48 h) > 1.36 μM (72 h) to 1.01 μM (96 h), respectively, against *L. acuminata*. The toxicological experiments to proof for environmental toxicity, if any, have also been carried out on the non-target freshwater fish *Channa punctatus* (Bloch) [Channidae (Ophicephalidae)], which shares the habitat with *L. acuminata*.

The sublethal doses of crotocaudin (40% and 80% of LC_{50}) administered over 24 h caused significant changes in the carbohydrate and nitrogenous metabolisms in nervous, hepatopancreas, and ovotestis tissues of *Lymnaea acuminata*. *Channa punctatus* was also exposed to sublethal doses of crotocaudin (40% and 80% of 24-h LC_{50} of *L. acuminata*) for 96 h which showed significant alterations in the metabolism in muscle, liver, and gonad tissues. After withdrawal of crotocaudin the snail tissues recovered in part after 7 days and the fish tissues completely.

Key words: Snail, Schistosomiasis, Metabolism, Enzyme Activity

Introduction

Although the freshwater snail *Lymnaea* (Radix) *acuminata* (Lamarck) is an intermediate host of the liver flukes *Fasciola hepatica* and *Fasciola gigantica*, which cause endemic fascioliasis in cattle and livestock, this snail is an important link in the detritus food chain of the aquatic ecosystem. Fascioliasis caused by *Fasciola hepatica* is common in sheep, cattle, goat, and other herbivorous animals throughout the world (Hyman, 1970; Agarwal and Singh, 1988). Singh and Agarwal (1981) reported that 94% of the buffaloes slaughtered in Gorakhpur, Uttar Pradesh, India were infected by the liver fluke *Fasciola gigantica*.

The method of controlling the schistosomiasis and fascioliasis is to kill the intermediate host snail with molluscicides (Singh and Agarwal, 1988). Widespread and heavy use of synthetic pesticides has been found to affect water bodies due to their high toxicity, bioaccumulation and long-term persistence (Marston and Hostettmann, 1987; Sleigh *et al*., 1985). The hazardous nature of synthetic pesticides has prompted the scientists to find less disruptive newer options in pest technologies. Molluscicides of plant origin are widely used, because the selective toxicity of these products is high, they are easily biodegradable in nature, safe for the user, and cheap (Marston and Hostettmann, 1985).

The aim of the present study was to report the molluscicidal activity of crotocaudin, extracted from the stem bark of *Croton tiglium*, against the harmful freshwater snail *Lymnaea acuminata.* Its effect on biochemical parameters of the target snail *L. acuminata* and the non-target freshwater fish *Channa punctatus* (Bloch) were also studied. *Channa punctatus* is a common fish of Indian fishery and shares the habitat with the snails in aquatic ecosystems.

Material and Methods

Plant material

The stem bark of the medicinal plant *Croton tiglium* was collected locally from the botanical

0939–5075/2010/0500–0327 $ 06.00

garden of D. D. U., Gorakhpur University, Gorakhpur, India and identified by Prof. S. K. Singh, Department of Botany, D. D. U., Gorakhpur University, Gorakhpur, India.

Extraction of crotocaudin from stem bark

Pure crotocaudin was isolated from the stem bark of *C. tiglium* by the method of Chatterjee and Banerjee (1977). The stem bark of *C. tiglium* was dried in an incubator at 37 °C. Then it was powdered with the help of mechanical device. 2 kg of the powder were extracted in a Soxhlet apparatus with petroleum ether for about 70 h, and 340 mL of solution were obtained. After evaporating the concentrated solution by a vacuum pump, the organic constituents present in the stem bark, crotocaudin, taraxerone-2, taraxerol, taraxeryl acetate-4, and sitosterol, were extracted with petroleum ether. Crotocaudin is soluble in organic solvents such as $CHCl_3$ and $CHCl_3$/MeOH. Identification of the isolated compound was confirmed with an authentic sample of crotocaudin ($C_{19}H_{18}O_5$), supplied by Sigma Chemical Co., St. Louis, USA.

The extracted compound was stored in an airtight desiccator. Toxicity experiments were performed by the method of Singh and Agarwal (1988). The freshwater snail *L. acuminata* was exposed to four different doses of crotocaudin: 0.92 μM, 1.53 μM, 2.15 μM, and 3.07 μM, respectively, for 24 h, 48 h, 72 h, and 96 h in an aquarium. Ten snails were kept in 3 L dechlorinated tap water. Mortality was recorded every 24 h up to 96 h. Control animals were kept under similar conditions without any treatment. Each set of experiments was replicated six times. Effective doses (LC values), upper and lower confidence limits, slope value, 't' ratio, 'g' factor, and heterogeneity were calculated by the Probit log analysis method using the POLO computer program of Russel *et al*. (1977).

To test the environmental toxicity, if any, the toxic effect of crotocaudin was also studied in mixed populations of target organisms (snails) and non-target organisms (fish). In this experiment, groups of 10 *L. acuminata* and 10 *C. punctatus* were put together in 3 L dechlorinated tap water and exposed to 31.09 μM (LC_{90} 24 h of *L. acuminata*) crotocaudin for 24 h.

Experimental conditions

Water temperature, pH, dissolved O_2, and total alkalinity were examined according to APHA (1992). Water temperature ranged from 27.4 to 28.6 °C. The other parameters were within the following ranges: total alkalinity, 43–62 ppm; pH, 6.8–7.7; and dissolved O_2, 7.8–10.3 mg/L.

Treatment protocol for dose-response relationship

The freshwater snail *Lymnaea acuminata* was kept in glass aquaria containing 3 L dechlorinated tap water. Each aquarium contained 30 experimental animals. *Lymnaea acuminata* was exposed for 24 h and *Channa punctatus* for 96 h to sublethal doses, 2.15 μM and 4.29 μM (40% and 80% of 24-h LC_{50} of *L. acuminata*), of the extracted compound crotocaudin. Control animals were held under similar conditions without any treatment. After completion of treatment the test animals were removed from aquaria and washed with freshwater. The nervous (NT), hepatopancreas (HP), and ovotestis (OT) tissues of *L. acuminata* and muscle, liver, and gonad tissues of *C. punctatus* were quickly dissected in an ice tray and used for biochemical analyses.

To observe the effect of withdrawal from treatment, *Lymnaea acuminata* was exposed for 24 h and *Channa punctatus* for 96 h to sublethal doses of crotocaudin, *i.e.* 4.29 μM (80% of 24 h LC_{50} of *L. acuminata*), and then were transferred to extract-free water. This water was changed every 24 h for the next 7 d, after which biochemical parameters were measured in the different snail and fish tissues. Each experiment was replicated at least six times and the values have been expressed as means ± SE of six replicates. Student's 't' test and analysis of variance were applied to locate significant changes (Sokal and Rohlf, 1973).

Biochemical estimation

Protein levels were estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Homogenates (5 mg mL^{-1}, w/v) were prepared in 10% TCA.

Estimation of total free amino acid was performed according to the method of Spies (1957). Homogenates (10 mg mL^{-1}, w/v) were prepared in 95% ethanol, centrifuged at 6000 × *g* and used for amino acid estimation.

Estimation of nucleic acids (DNA and RNA) was performed according to Schneider (1957) using diphenylamine and orcinol reagents, respectively. Homogenates (1 mg mL^{-1}, w/v) were prepared in 5% TCA at 90 °C and centrifuged at 5000 × *g* for 20 min. The supernatant was used for estimation. Both DNA and RNA levels have been expressed as μg mg^{-1} tissue.

Glycogen was estimated by the anthrone method of Van Der Vies (1954), modified by Mahendru and Agarwal (1982) for the snail *L. acuminata*. In the present experiment 50 mg of tissue were homogenized with 5 mL of cold 5% TCA. The homogenate was filtered and 1.0 mL of filtrate was used for the assay.

The pyruvate level was measured according to Friedemann and Haugen (1943). Homogenate (50 mg mL^{-1}, w/v) was prepared in 10% TCA. Sodium pyruvate was taken as standard.

Lactate was estimated according to Barker and Summerson (1941), modified by Huckabee (1961). Homogenate (50 mg mL^{-1}, w/v) was prepared in 10% cold TCA. Sodium lactate was taken as standard.

The protease activity was estimated by the method of Moore and Stein (1954). Homogenate (50 mg mL^{-1}, w/v) was prepared in cold distilled water. The optical density was measured at 570 nm. The enzyme activity was expressed in μmol of tyrosine equivalents mg $protein^{-1}$ h^{-1}.

The activities of acid and alkaline phosphatase were measured by the method of Bergmeyer (1967), modified by Singh and Agarwal (1983). Tissue homogenates (2% w/v) were prepared in ice-cold 0.9% saline and centrifuged at 5000 × *g* and 0 °C for 15 min. The optical density was measured at 420 nm against a blank, prepared simultaneously. The enzyme activities have been expressed as amount of *p*-nitrophenol formed 30 min^{-1} mg $protein^{-1}$.

The lactic dehydrogenase activity (LDH) was measured by the method of Sigma Diagnostics (1984). Homogenates (50 mg mL^{-1}, w/v) were prepared in 1 mL of 0.1 M phosphate buffer, pH 7.5, for 5 min in an ice bath. The enzyme activity has been expressed as μmol of pyruvate reduced min^{-1} mg $protein^{-1}$.

The succinic dehydrogenase activity (SDH) was measured by the method of Arrigoni and Singer (1962). Homogenate (50 mg mL^{-1}, w/v) was prepared in 1 mL of 0.5 M potassium phosphate buffer, pH 7.6, for 5 min in an ice bath. The optical density was measured at 600 nm. The enzyme activity has been expressed as μmol of dye reduced min^{-1} mg $protein^{-1}$.

The cytochrome oxidase activity was measured according to the method of Cooperstein and Lazarow (1951). Homogenates (50 mg mL^{-1}, w/v) were prepared in 1 mL of 0.33 M phosphate buffer, pH 7.4, for 5 min in ice bath. The enzyme activity has been expressed in arbitrary units min^{-1} mg $protein^{-1}$.

Acetylcholinesterase (AchE) was estimated by the method of Ellman *et al.* (1961). Homogenate (50 mg mL^{-1}) was prepared in 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 °C. Protein estimation was done by the Method of Lowry *et al.* (1951). The optical density was measured at 412 nm at 25 °C. The enzyme activity was expressed in μmol sulfohydryl min^{-1} mg $protein^{-1}$.

Results

Molluscicidal activity

The toxicity of the extracted compound crotocaudin (Fig. 1) was time- as well as dose-dependent for the freshwater snail *Lymnaea acuminata.* There was a significant negative correlation between LC_{50} values and the exposure periods (Table I). Thus, with an increase in exposure time, the LC_{50} decreased from 5.37 μM (24 h) > 2.08 μM (48 h) > 1.36 μM (72 h) > to 1.01 μM (96 h).

The active compound crotocaudin, which was effective against the freshwater snail *L. acumi-*

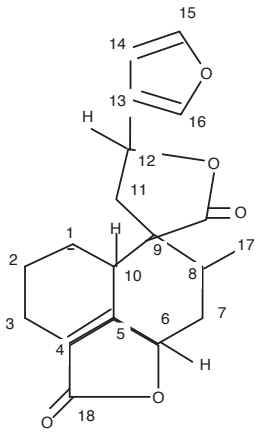

Fig. 1. Chemical structure of crotocaudin.

Table I. Toxicity (LC_{10}, LC_{50}, and LC_{90}) of crotocaudin against the freshwater snail *Lymnaea acuminata* at different time intervals.

Exposure time [h]	Effective dose [μM]	LCL[a] [μM]	UCL[a] [μM]	Slope value	'g' factor	't' ratio	Hetero-geneity
24	LC_{10} 0.92	0.313	1.306				
	LC_{50} 5.37	3.493	25.200	1.684 ± 0.506	0.34	3.32	0.34
	LC_{90} 31.09	10.843	17.442				
48	LC_{10} 0.66	0.374	0.886				
	LC_{50} 2.08	1.791	2.502	2.583 ± 0.462	0.17	5.58	0.17
	LC_{90} 6.51	4.563	13.281				
72	LC_{10} 0.49	0.276	0.678				
	LC_{50} 1.36	1.138	1.564	2.909 ± 0.466	0.09	6.24	0.20
	LC_{90} 3.76	2.996	5.630				
96	LC_{10} 0.52	0.353	0.656				
	LC_{50} 1.01	0.849	1.131	4.476 ± 0.637	0.07	7.03	0.66
	LC_{90} 1.95	1.720	2.336				

Batches of ten snails were exposed to four different concentrations of crotocaudin.
Final concentrations (w/v) in aquarium water are given.
The regression coefficient showed that there was significant ($P < 0.05$) negative correlation between exposure time and different LC values.
[a] LCL, lower confidence limit; UCL, upper confidence limit.

Table II. Changes in total protein, total free amino acid, and nucleic acid (DNA and RNA) levels (μg mg^{-1}), and activities of protease (μmol of tyrosine equivalents mg $protein^{-1}$ h^{-1}) and acid and alkaline phosphatase (amount of *p*-nitrophenol formed 30 min^{-1} mg $protein^{-1}$) in nervous (NT), hepatopancreas (HP), and ovotestis (OT) tissues of *Lymnaea acuminata* after exposure to sublethal doses of 40% and 80% (2.15 μM and 4.29 μM) crotocaudin for 24 h and 7 days after withdrawal.

Parameter	Tissue	Control	40% of LC_{50} (24 h) (2.15 μM)	80% of LC_{50} (24 h) (4.29 μM)	7 days after withdrawal
Protein	NT	60.0 ± 0.02 (100)	28.80 ± 0.27 (48)	16.80 ± 0.02[a] (28)	57.00 ± 0.58[a] (95)
	HP	64.2 ± 2.20 (100)	27.60 ± 0.80 (43)	21.82 ± 0.78 (34)	61.63 ± 1.00[a] (96)
	OT	60.1 ± 0.37 (100)	26.44 ± 0.65 (44)	15.02 ± 0.81 (25)	58.89 ± 0.31[a] (98)
Amino acid	NT	32.3 ± 0.25 (100)	52.03 ± 1.13 (161)	53.94 ± 0.22 (167)	33.59 ± 0.20[a] (104)
	HP	27.8 ± 0.12 (100)	39.75 ± 0.35 (143)	41.42 ± 1.01 (149)	29.46 ± 0.33[a] (106)
	OT	33.2 ± 0.08 (100)	54.44 ± 0.13 (164)	55.44 ± 0.11 (167)	35.85 ± 0.04[a] (108)
DNA	NT	75.6 ± 1.10 (100)	43.84 ± 0.32 (58)	28.72 ± 0.18 (38)	72.57 ± 0.60[a] (96)
	HP	72.0 ± 2.10 (100)	49.68 ± 0.35 (69)	24.48 ± 0.21 (34)	68.40 ± 0.48[a] (95)
	OT	82.3 ± 3.00 (100)	42.79 ± 0.18 (52)	24.69 ± 0.48 (30)	80.65 ± 0.32[a] (98)
RNA	NT	52.32 ± 1.00 (100)	26.68 ± 0.21 (51)	18.83 ± 0.28 (36)	51.79 ± 0.21[a] (99)
	HP	50.22 ± 2.00 (100)	32.64 ± 0.67 (65)	15.06 ± 0.16 (30)	48.71 ± 0.13[a] (97)
	OT	53.32 ± 2.23 (100)	25.59 ± 2.10 (48)	13.86 ± 0.51 (26)	50.65 ± 0.12[a] (95)
Protease	NT	0.368 ± 0.002 (100)	0.522 ± 0.041 (142)	0.563 ± 0.046[a] (153)	0.404 ± 0.058[a] (110)
	HP	0.378 ± 0.003 (100)	0.521 ± 0.002 (138)	0.585 ± 0.002[a] (155)	0.396 ± 0.013[a] (105)
	OT	0.360 ± 0.007 (100)	0.525 ± 0.004 (146)	0.569 ± 0.003[a] (158)	0.388 ± 0.012[a] (108)
Acid phosphatase	NT	0.265 ± 0.006 (100)	0.247 ± 0.004 (93)	0.201 ± 0.005[a] (76)	0.249 ± 0.102[a] (94)
	HP	0.277 ± 0.005 (100)	0.268 ± 0.009 (97)	0.225 ± 0.008[a] (81)	0.254 ± 0.003[a] (92)
	OT	0.281 ± 0.008 (100)	0.266 ± 0.006 (95)	0.219 ± 0.003[a] (78)	0.258 ± 0.005[a] (92)
Alkaline phosphatase	NT	0.465 ± 0.020 (100)	0.362 ± 0.002 (78)	0.306 ± 0.003[a] (66)	0.446 ± 0.005[a] (96)
	HP	0.460 ± 0.005 (100)	0.327 ± 0.001 (71)	0.285 ± 0.001[a] (62)	0.447 ± 0.003[a] (97)
	OT	0.466 ± 0.007 (100)	0.372 ± 0.003 (80)	0.312 ± 0.006[a] (67)	0.456 ± 0.002[a] (98)

[a] Significant ($P < 0.05$) Student's 't' test was applied between 80% of LC_{50} (24 h) and withdrawal groups. Values are means ± SE of six replicates. Values in parentheses are percent changes with control taken as 100%.

Table III. Changes in glycogen (mg g^{-1}), pyruvate (μmol g^{-1}), and lactate (mg g^{-1}) level, and activity of LDH (μmol pyruvate reduced min^{-1} mg $protein^{-1}$), SDH (μmol of dye reduced min^{-1} mg $protein^{-1}$), cytochrome oxidase (arbitrary units min^{-1} mg $protein^{-1}$) and AChE (μmol sulfohydryl min^{-1} mg $protein^{-1}$) after 24 h of exposure to sublethal doses of 40% and 80% (2.15 μM and 4.29 μM) crotocaudin in nervous (NT), hepatopancreas (HP), and ovotestis (OT) tissues of *Lymnaea acuminata* and 7 days after withdrawal.

Parameter	Tissue	Control	40% of LC_{50} (24 h) (2.15 μM)	80% of LC_{50} (24 h) (4.29 μM)	7 days after withdrawal
Glycogen	NT	5.8 ± 0.02 (100)	2.5 ± 0.27 (44)	1.74 ± 0.02[a] (30)	5.45 ± 0.58[a] (94)
	HP	6.2 ± 2.20 (100)	3.1 ± 0.80 (50)	2.48 ± 0.78 (40)	6.01 ± 1.00[a] (97)
	OT	8.4 ± 0.37 (100)	3.8 ± 0.65 (46)	2.85 ± 0.81 (34)	7.81 ± 0.31[a] (93)
Pyruvate	NT	0.678 ± 0.02 (100)	0.223 ± 0.07 (33)	0.183 ± 0.25 (27)	0.610 ± 0.21[a] (90)
	HP	0.610 ± 0.05 (100)	0.250 ± 0.24 (41)	0.244 ± 0.10 (40)	0.567 ± 0.08[a] (93)
	OT	0.567 ± 0.12 (100)	0.158 ± 0.15 (28)	0.147 ± 0.12 (26)	0.521 ± 0.02[a] (92)
Lactate	NT	3.11 ± 0.06 (100)	5.22 ± 0.18 (168)	5.69 ± 0.17 (183)	3.60 ± 0.02[a] (116)
	HP	2.88 ± 0.02 (100)	5.21 ± 0.06 (181)	5.47 ± 0.01[a] (190)	3.25 ± 0.05[a] (113)
	OT	4.01 ± 0.08 (100)	6.41 ± 0.73 (160)	7.41 ± 0.07 (185)	4.69 ± 0.01[a] (117)
LDH	NT	0.080 ± 0.220 (100)	0.062 ± 0.002 (78)	0.040 ± 0.001[a] (50)	0.072 ± 0.003[a] (91)
	HP	0.090 ± 0.160 (100)	0.078 ± 0.005 (87)	0.054 ± 0.006[a] (60)	0.081 ± 0.006[a] (90)
	OT	0.83 ± 0.110 (100)	0.069 ± 0.001 (84)	0.040 ± 0.004[a] (49)	0.077 ± 0.001[a] (93)
SDH	NT	17.23 ± 0.05 (100)	22.74 ± 0.21 (132)	30.49 ± 0.37 (177)	19.62 ± 0.27[a] (114)
	HP	16.20 ± 0.03 (100)	19.44 ± 0.18 (120)	24.78 ± 0.18 (153)	17.82 ± 0.12[a] (110)
	OT	16.67 ± 0.01 (100)	21.33 ± 0.30 (128)	27.67 ± 0.31 (166)	19.17 ± 1.13[a] (115)
Cytochrome oxidase	NT	17.33 ± 0.018 (100)	12.47 ± 0.130 (72)	13.17 ± 0.005[a] (76)	16.29 ± 0.102[a] (94)
	HP	15.22 ± 0.011 (100)	14.76 ± 0.009 (97)	12.32 ± 0.008[a] (81)	14.00 ± 0.003[a] (92)
	OT	17.11 ± 0.027 (100)	0.266 ± 0.006 (95)	16.25 ± 0.003[a] (78)	15.74 ± 0.005[a] (92)
AChE	NT	0.465 ± 0.200 (100)	0.362 ± 0.002 (78)	0.306 ± 0.003[a] (66)	0.446 ± 0.005[a] (96)
	HP	0.460 ± 0.050 (100)	0.327 ± 0.001 (71)	0.285 ± 0.001[a] (62)	0.447 ± 0.003[a] (97)
	OT	0.466 ± 0.070 (100)	0.372 ± 0.003 (80)	0.312 ± 0.006[a] (67)	0.456 ± 0.002[a] (98)

Details are as given in Table II.

nata, would also cause death amongst the fish at higher doses. Consequently, a mixed population of 10 snails (*L. acuminata*) and 10 fishes (*C. punctatus*) was treated with the LC_{90} (24 h) of crotocaudin up to 96 h; there was no mortality amongst the fish *C. punctatus*.

Effect on freshwater target snail

Data of sublethal doses of 40% and 80% of LC_{50} (2.15 μM and 4.29 μM) of crotocaudin exposure to the freshwater snail *L. acuminata* are given in Tables II and III. Exposure of snails to sublethal doses of crotocaudin for 24 h caused significant alterations in the nitrogenous and carbohydrate metabolism in different body tissues of the freshwater snail *L. acuminata.* Total protein and nucleic acids (DNA and RNA) levels were significantly reduced, while the free amino acid level was significantly enhanced in all body tissues after exposure to sublethal doses. Acid and alkaline phosphatase activities were significantly reduced, while the protease activity was increased after exposure.

Total protein levels were reduced to 28%, 34%, and 25% of controls after exposure to sublethal doses of 4.29 μM crotocaudin in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*, respectively. The DNA level was reduced to 38%, 34%, and 30% of controls after treatment with 4.29 μM crotocaudin in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*, respectively. The RNA level was reduced to 36%, 30%, and 26% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, the in nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. Total free amino acid levels were induced to 167%, 149%, and 167% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata* (Table II).

The activity of acid phosphatase was inhibited to 76%, 81%, and 78% of controls after treat-

Table IV. Changes in total protein, total free amino acid, and nucleic acid (DNA and RNA) levels (μg mg^{-1}), and activities of protease (μmol of tyrosine equivalents mg protein^{-1} h^{-1}) and acid and alkaline phosphatase (amount of *p*-nitrophenol formed 30 min^{-1} mg protein^{-1}) in muscle, liver, and gonad tissues of *Channa punctatus* after 96 h of exposure to sublethal doses of 40% and 80% (2.15 μM and 4.29 μM) crotocaudin and 7 days after withdrawal.

Parameter	Tissue	Control	40% of LC_{50} (24 h) (2.15 μM)	80% of LC_{50} (24 h) (4.29 μM)	7 days after withdrawal
Protein	Muscle	166.2 ± 0.72 (100)	104.7 ± 0.21 (63)	63.1 ± 0.33 (38)	159.5 ± 0.32[a] (96)
	Liver	148.1 ± 0.61 (100)	124.4 ± 0.32 (84)	119.9 ± 0.21 (81)	145.1 ± 1.12[a] (98)
	Gonad	151.4 ± 0.45 (100)	121.1 ± 0.16 (80)	75.60 ± 0.77 (50)	139.2 ± 0.58[a] (92)
Amino acid	Muscle	37.21 ± 0.22 (100)	41.30 ± 0.27 (111)	48.00 ± 0.31 (129)	42.04 ± 0.32[a] (113)
	Liver	23.30 ± 0.30 (100)	29.59 ± 0.41 (127)	34.01 ± 0.38 (146)	24.93 ± 0.22[a] (107)
	Gonad	38.00 ± 0.83 (100)	49.40 ± 0.06 (130)	57.38 ± 0.05 (151)	44.08 ± 0.06[a] (116)
DNA	Muscle	151.41 ± 0.72 (100)	121.12 ± 0.13 (80)	92.36 ± 0.18 (61)	146.8 ± 0.71[a] (97)
	Liver	149.01 ± 0.68 (100)	128.14 ± 0.06 (86)	108.77 ± 0.11 (73)	138.5 ± 0.47[a] (93)
	Gonad	144.02 ± 0.75 (100)	115.21 ± 0.41 (80)	86.41 ± 0.22 (60)	132.4 ± 0.28[a] (92)
RNA	Muscle	104.00 ± 0.21 (100)	87.36 ± 0.02 (84)	70.72 ± 0.03 (68)	102.9 ± 0.051[a] (99)
	Liver	100.00 ± 0.32 (100)	91.00 ± 0.06 (91)	76.00 ± 0.16 (76)	94.00 ± 0.017[a] (94)
	Gonad	107.00 ± 0.66 (100)	84.53 ± 0.38 (79)	67.41 ± 0.21 (63)	99.51 ± 0.153 (93)
Protease	Muscle	0.565 ± 0.013 (100)	0.785 ± 0.043 (139)	0.887 ± 0.011[a] (157)	0.531 ± 0.053[a] (94)
	Liver	0.651 ± 0.018 (100)	0.807 ± 0.017 (124)	0.911 ± 0.018[a] (140)	0.631 ± 0.009[a] (97)
	Gonad	0.600 ± 0.010 (100)	0.780 ± 0.011 (130)	0.942 ± 0.013[a] (157)	0.558 ± 0.141[a] (93)
Acid phosphatase	Muscle	0.311 ± 0.016 (100)	0.099 ± 0.019 (32)	0.090 ± 0.012[a] (29)	0.295 ± 0.019[a] (95)
	Liver	0.296 ± 0.013 (100)	0.103 ± 0.016 (35)	0.076 ± 0.005[a] (26)	0.275 ± 0.101[a] (93)
	Gonad	0.278 ± 0.018 (100)	0.091 ± 0.015 (33)	0.066 ± 0.013[a] (24)	0.266 ± 0.003[a] (96)
Alkaline phosphatase	Muscle	0.450 ± 0.005 (100)	0.198 ± 0.003 (44)	0.130 ± 0.005[a] (29)	0.405 ± 0.008[a] (90)
	Liver	0.400 ± 0.028 (100)	0.152 ± 0.008 (38)	0.104 ± 0.003[a] (26)	0.356 ± 0.002[a] (89)
	Gonad	0.437 ± 0.011 (100)	0.214 ± 0.003 (49)	0.139 ± 0.007[a] (32)	0.380 ± 0.009[a] (87)

Details are as given in Table II.

ment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue. The activity of alkaline phosphatase was reduced to 66%, 62%, and 67% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas and ovotestis tissue. The protease activity was increased to 153%, 155%, and 158% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata* (Table II).

Glycogen and pyruvate levels were significantly reduced, while the lactate level was significantly enhanced after exposure to sublethal doses in all body tissues. Lactic dehydrogenase (LDH), cytochrome oxidase, and acetylcholinesterase (AChE) activities were significantly reduced, while the succinic dehydrogenase (SDH) activity was increased after exposure.

The glycogen level was reduced to 30%, 40%, and 34% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. The pyruvate level was reduced to 27%, 40%, and 26% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. The lactate level was increased to 183%, 190%, and 185% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata* (Table III).

The lactic dehydrogenase activity was reduced to 50%, 60%, and 49% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. The activity of cytochrome oxidase was reduced to 76%, 81%, and 78% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. The acetylcholinesterase activity was reduced to 66%, 62%, and 67% of controls after treatment with sublethal doses of 4.29 μM croto-

Table V. Changes in glycogen (mg g^{-1}), pyruvate (μmol g^{-1}), and lactate (mg g^{-1}) levels, and activities of LDH (μmol pyruvate reduced min^{-1} mg $protein^{-1}$), SDH (μmol of dye reduced min^{-1} mg $protein^{-1}$), cytochrome oxidase (arbitrary units min^{-1} mg $protein^{-1}$), and AChE (μmol sulfohydryl min^{-1} mg $protein^{-1}$) in muscle, liver, and gonad tissues of *Channa punctatus* after 96 h of exposure to sublethal doses of 40% and 80% (2.15 μM and 4.29 μM) crotocaudin and 7 days after withdrawal.

Parameter	Tissues	Control	40% of LC_{50} (24 h) (2.15 μM)	80% of LC_{50} (24 h) (4.29 μM)	7 days after withdrawal
Glycogen	Muscle	2.21 ± 0.02 (100)	1.83 ± 0.03 (83)	1.37 ± 0.06 (62)	1.94 ± 0.01[a] (88)
	Liver	2.37 ± 0.01 (100)	1.82 ± 0.16 (77)	1.56 ± 0.01 (66)	1.99 ± 0.02[a] (84)
	Gonad	3.00 ± 0.22 (100)	2.55 ± 0.04 (85)	2.10 ± 0.03[a] (70)	2.70 ± 0.05[a] (90)
Pyruvate	Muscle	3.981 ± 0.012 (100)	2.030 ± 0.017 (51)	1.313 ± 0.018[a] (33)	3.622 ± 0.011[a] (91)
	Liver	3.072 ± 0.015 (100)	1.90 ± 0.031 (62)	1.198 ± 0.005[a] (39)	1.351 ± 0.028[a] (44)
	Gonad	2.962 ± 0.016 (100)	1.65 ± 0.011 (56)	0.888 ± 0.023[a] (30)	2.72 ± 0.015[a] (92)
Lactate	Muscle	4.012 ± 0.010 (100)	5.33 ± 0.088 (133)	7.26 ± 0.050[a] (181)	4.61 ± 0.068[a] (115)
	Liver	3.037 ± 0.015 (100)	4.22 ± 0.018 (139)	5.22 ± 0.069[a] (172)	3.37 ± 0.085[a] (111)
	Gonad	4.111 ± 0.071 (100)	5.30 ± 0.081 (129)	6.90 ± 0.100 (168)	4.39 ± 0.038[a] (107)
LDH	Muscle	411.2 ± 0.77 (100)	553.5 ± 0.80 (86)	254.9 ± 0.86 (62)	374.1 ± 0.17[a] (91)
	Liver	530.1 ± 2.10 (100)	503.5 ± 0.36 (95)	365.7 ± 0.78 (69)	498.2 ± 0.77[a] (94)
	Gonad	450.2 ± 0.81 (100)	418.6 ± 0.65 (93)	342.1 ± 0.69 (76)	423.1 ± 0.86[a] (94)
SDH	Muscle	63.50 ± 0.22 (100)	79.37 ± 0.21 (125)	87.63 ± 0.22 (138)	71.12 ± 0.16[a] (112)
	Liver	61.00 ± 0.16 (100)	72.59 ± 0.31 (119)	86.01 ± 0.18 (141)	69.54 ± 0.15[a] (114)
	Gonad	65.10 ± 0.25 (100)	72.91 ± 0.14 (112)	98.30 ± 0.11 (151)	69.65 ± 0.21[a] (107)
Cytochrome oxidase	Muscle	27.22 ± 0.20 (100)	22.86 ± 0.15 (84)	16.33 ± 0.21 (60)	25.85 ± 0.28[a] (95)
	Liver	24.11 ± 0.04 (100)	23.38 ± 0.20 (97)	15.91 ± 0.16 (66)	22.18 ± 0.22[a] (92)
	Gonad	35.01 ± 0.06 (100)	30.10 ± 0.09 (86)	25.20 ± 0.10 (72)	32.55 ± 0.19[a] (93)
AChE	Muscle	0.089 ± 0.001 (100)	0.045 ± 0.002 (51)	0.034 ± 0.006[a] (39)	0.084 ± 0.003[a] (95)
	Liver	0.091 ± 0.008 (100)	0.050 ± 0.006 (56)	0.032 ± 0.005[a] (36)	0.083 ± 0.001[a] (92)
	Gonad	0.081 ± 0.020 (100)	0.038 ± 0.001 (48)	0.018 ± 0.003[a] (23)	0.076 ± 0.012[a] (94)

Details are as given in Table II.

caudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata*. The succinic dehydrogenase activity was increased to 177%, 153%, and 166% of controls after treatment with sublethal doses of 4.29 μM crotocaudin, respectively, in the nervous, hepatopancreas, and ovotestis tissue of *L. acuminata* (Table III).

Effect on freshwater non-target fish

Higher doses (LC_{90} of snails) have no apparent toxic effect on the non-target freshwater fish *C. punctatus* after 24 h of exposure. But exposure of fishes to sublethal doses, 2.15 μM and 4.29 μM (40% and 80% of 24-h LC_{50} of snail) of crotocaudin for 96 h caused a significant alteration in the nitrogenous and carbohydrate metabolism in different body tissues of *C. punctatus* (Tables IV and V).

Total protein and nucleic acids (DNA and RNA) levels were significantly reduced, while the free amino acid level was significantly enhanced after exposure to sublethal doses in all studied body tissues. Acid and alkaline phosphatase activities were significantly reduced, while the protease activity was increased after exposure. Total protein levels were reduced to 38%, 81%, and 50%, DNA level was reduced to 61%, 73%, and 60%, and the RNA level was reduced to 68%, 76%, and 63% in muscle, liver, and gonad tissue of *C. punctatus.* Total free amino acid levels increased to 129%, 146%, and 151% of controls after 96 h of treatment with 4.29 μM crotocaudin in muscle, liver, and gonad tissue, respectively (Table IV).

The activity of acid phosphatase was inhibited to 29%, 26%, and 24%, the activity of alkaline phosphatase was reduced to 29%, 26%, and 32% and the protease activity was increased to 157%, 140%, and 157% of controls after 96 h of treatment with 4.29 μM crotocaudin in muscle, liver and gonad tissue of *C. punctatus*, respectively (Table IV).

Glycogen and pyruvate levels were significantly reduced, while the lactate level was significantly enhanced in the studied body tissues after exposure to sublethal doses. Lactic dehydrogenase

(LDH), cytochrome oxidase and acetylcholinesterase (AChE) activities were significantly reduced, while the succinic dehydrogenase (SDH) activity was increased after exposure. The glycogen level was reduced to 62%, 66%, and 70% and the pyruvate level was reduced to 33%, 39%, and 30% in muscle, liver and gonad tissue of *C. punctatus*. The lactate level was increased to 181%, 172%, and 168% of controls after 96 h of treatment with 4.29 μM crotocaudin in muscle, liver, and gonad tissue of *C. punctatus*, respectively (Table V).

The lactic dehydrogenase activity was reduced to 62%, 69%, and 76%, the activity of cytochrome oxidase was reduced to 60%, 66%, and 72%, and the acetylcholinesterase activity was reduced to 39%, 36%, and 23% in muscle, liver and gonad tissue of *C. punctatus*, respectively. The succinic dehydrogenase activity was increased to 138%, 141%, and 151% of controls after 96 h of treatment with 4.29 μM crotocaudin in muscle, liver, and gonad tissue of *C. punctatus*, respectively (Table V).

Discussion and Conclusion

It is evident from the results presented here that the extracted compound crotocaudin is toxic to *Channa punctatus* at higher concentrations and longer exposure periods. The exposure to 40% and 80% of snail LC_{50} for 24 h did not cause any significant changes in the level of carbohydrate and nitrogenous metabolism of fish tissues, while this treatment continued up to 96 h decreased the carbohydrate and nitrogenous metabolism levels significantly.

The depletion of the protein fraction in different tissues of snails and fishes may have been due to their degradation and possible utilization of degraded products for metabolic purposes. Mommensen and Walsh (1992) reported that proteins, which are the main source of the nitrogenous metabolism, are mainly involved in the architecture of the cell and during chronic periods of stress they are also a source of energy. Increment in the free amino acids level was the result of breakdown of protein for energy requirement and impaired incorporation of amino acids in protein synthesis. Inhibition of DNA synthesis might affect protein as well as amino acid levels by decreasing the level of RNA in the protein synthesis machinery (Nordenskjold *et al.*, 1979).

However, in any tissue total depletion of glycogen will not occur, because it would result in the disruption of enzyme systems associated with the carbohydrate metabolism (Heilmeyer *et al.*, 1970), since the enzyme systems are associated with glycolysis and TCA cycle from a constitutive enzyme system (Woodward and Woodward, 1978).

Carbohydrates are the primary and immediate source of the metabolism (Arasta *et al.*, 1996). Suggesting that, in stress conditions, carbohydrate reserves deplete to meet energy demand, thus depletion of glycogen may be due to direct utilization for energy generation, a demand caused by active moiety-induced hypoxia. The glycogenolysis seems to be the result of increased secretion of catecholamine due to stress. A decrease in the pyruvate level is due to higher energy demand during exposure, which suggests the possibility of a shift towards anaerobic dependence due to a remarkable drop for oxygen. The level of tissue lactic acid is known to act as an index of anaerobiosis which might be beneficial to the animal to tolerate hypoxic conditions (Thoye, 1971). The increase in lactate also suggests a shift towards anaerobiosis because of hypoxia leading to respiratory distress (Siva Prasada Rao, 1980).

Lactic dehydrogenase catalyzes the interconversions of lactic acid and pyruvic acid during anaerobic conditions. Inhibition of lactic dehydrogenase and cytochrome oxidase activity shows that crotocaudin significantly inhibits the aerobic as well as anaerobic metabolism in exposed animals (Everse and Kalpan, 1973). Succinic dehydrogenase is one of the active regulatory enzymes of the TCA cycle. Inhibition of cytochrome oxidase activity by crotocaudin supports that Euphorbiales show a profound impact on the oxidative metabolism.

Withdrawal experiments were performed to see whether biochemical alteration caused by crotocaudin would return to normal, if the treatment ends. There was nearly complete recovery of the total protein, total free amino acid, lactate, nucleic acid (DNA and RNA), and pyruvate level, the activity of cytochrome oxidase, succinic dehydrogenase, protease, lactic dehydrogenase, acetylcholinesterase, and acid and alkaline phosphatase, and a partial recovery of the glycogen level in the different body tissues of the freshwater snail *L. acuminata* and the fish *C. punctatus*.

The statistical data analysis of the toxicity brought out several important points. The χ^2 test

for goodness of fit (heterogeneity) demonstrated that the mortality counts were not found to be significantly heterogeneous and other variables (*e.g.* resistance) did not significantly affect the LC_{50} values, as these were found to lie within the 95% confidence limit. The regression test ('t' ratio) was greater than 1.96, and the potency estimation test ('g' factor) was less than 0.5 at all probability levels. The slope is, thus, an index of the susceptibility of the target animal to the extracted compound used. A steep slope is also indicative of rapid absorption and onset of effects. Since the LC_{50} value of crotocaudin is within the 95% confidence limit, it is obvious that in replicate test of random samples, the concentration response lines, would fall in the same range (Rand and Petrocelli, 1988).

It is believed that the extracted compound crotocaudin may be used as a potent source of molluscicides; plant products are less expensive, easily available, easily soluble in water, and more safe for the non-target animals than synthetic molluscicides.

Acknowledgements

R. P. Yadav is thankful to Council of Scientific and Industrial Research, Government of India.

Agarwal R. A. and Singh D. K. (1988), Harmful gastropods and their control. Acta Hydrochim. Hydrobiol. **16**, 113–138.

APHA (1992), Standard methods for the examination of water and waste water. American Public Health Association, Washington, DC, USA, pp. 1268–1273.

Arasta T., Bais V. S., and Thakur P. (1996), Effect of nuvan on some biochemical parameters of Indian catfish *Mystus vittatus*. Environ. Biol. **17**, 167–169.

Arrigoni O. and Singer T. P. (1962), Limitations of the phenazine methosulphate assay for succinic and related dehydrogenases. Nature **193**, 1256–1258.

Barker S. B. and Summerson W. H. (1941), The colorimetric determination of lactic acid in biological materials. J. Biol. Chem. **138**, 535–542.

Bergmeyer U. H. (1967), Method of Enzymatic Analysis. Academic Press, New York, p. 1129.

Chatterjee A. and Banerjee A. (1977), Crotocaudin, a rearranged labdane type norditerpene from *Croton caudatus* Geisel. Tetrahedron **33**, 2407–2414.

Cooperstein S. J. and Lazarow A. (1951), Microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem. **189**, 665–670.

Ellman G. L., Courtney K. D., Andres V. J. R., and Featherstone R. M. (1961), A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. **7**, 88–95.

Everse T. and Kalpan N. O. (1973), Lactate dehydrogenase: Structure and function. In: Advances in Enzymology (Meister A., ed.). John Wiley and Sons, New York, pp. 61–133.

Friedemann T. E. and Haugen G. F. (1943), Pyruvic acid. I. Collection of blood for the determination of pyruvic acid and lactic acid. J. Biol. Chem. **144**, 67–77.

Heilmeyer L. M. G., Meyer F., Hascchke R. H., and Fischer E. H. (1970), Control of phosphorylase activity in a muscle glycogen particle II. Activation by calcium. J. Biol. Chem. **245**, 6649–6656.

Huckabee W. E. (1961), Blood analysis, determination of lactic acid. In: Hawk's Physiological Chemistry, 14th ed. (Oser B. L., ed.). Tata McGraw Hill, New Delhi, p. 1103.

Hyman L. H. (1970), The Invertebrates, Vol. Mollusca I. McGraw Hill, New York.

Lowry O. H., Rosenbrough N. J., Farr A. L., and Randall R. J. (1951), Protein measurement with folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Mahendru V. K. and Agarwal R. A. (1982), Changes induced by phorate in the carbohydrate metabolism of snail *Lymnaea acuminata*. Pest Sci. **13**, 611–616.

Marston A. and Hostettmann K. (1985), Plant molluscicides. Phytochemistry **24**, 639–652.

Marston A. and Hostettmann K. (1987), Antifungal, molluscicidal and cytotoxic compounds from plants used in traditional medicine. In: Bioactive Natural Products (Hostettmann K. and Lea P. J., eds.). Clarendon Press, Oxford, pp. 65–83.

Mommensen T. P. and Walsh P. J. (1992), Biochemical and environmental perspectives on nitrogen metabolism in fishes. Experientia **48**, 583–593.

Moore S. and Stein W. H. (1954), A modified ninhydrine reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. **211**, 907–913.

Nordenskjold M., Soderhall S., and Moldeus P. (1979), Studies of DNA strand induced in human fibroblasts by chemical mutagens/carcinogens. Mutat. Res. **63**, 393–400.

Rand G. M. and Petrocelli S. R. (1988), Fundamentals of Aquatic Toxicology. Hemisphere Publishing Corporation, New York, pp. 1129–1133.

Russel R. M., Robertson J. L., and Sevin N. E. (1977), A new computer programme for probit log analysis. Bull. Entomol. Soc. America **23**, 209–213.

Schneider W. C. (1957), Determination of nucleic acids in tissue by pentose analysis. In: Methods in Enzymology (Colowick S. P. and Kaplan N. O., eds.). Academic Press, New York, p. 680.

Sigma Diagnostics (1984), TM: Lactatic dehydrogenase (quantitative, colorimetric determination in serum, urine and cerebrospinal fluid) at 400–450 nm. Procedure no. 500. Sigma, St. Louis, USA. Aldrich-India (Survey 31/1), Sitharamapalaya, Mahadevapur Bangalore, India.

Singh O. and Agarwal R. A. (1981), Toxicity of certain pesticides to two economic species of snails in Northern India. J. Econ. Entomol. **74**, 568–571.

Singh D. K. and Agarwal R. A. (1983), *In vivo* and *in vitro* studies on synergism with anticholinesterase pesticides in the snail *Lymnaea acuminata*. Arch. Environ. Contam. Toxicol. **12**, 483–487.

Singh A. and Agarwal R. A. (1988), Possibility of using latex of euphorbiales for snail control. Sci. Total Environ. **77**, 231–236.

Siva Prasada Rao K. (1980), Studies on some aspects of metabolic changes with emphasis on carbohydrate utility in the cell-free systems of the teleost *T. mossambica* (Peters) under methyl parathion exposure. Ph.D. thesis, SV University, Tirupati, India.

Sleigh A. C., Mott K. E., Hoff R., Barreto M. L., Mota K. E., Maguire J. H., Sherlock I., and Weller T. H. (1985), Three year prospective study of the evolution of manson's schistosomiasis in North-East Brazil. Lancet **326**, 63–66.

Sokal R. R. and Rohlf F. J. (1973), Introduction to Biostatistics. W. H. Freeman Co., San Francisco, p. 368.

Spies J. R. (1957), Colorimetric procedure for amino acids. In: Methods in Enzymology (Colowick S. P. and Kalpan N. O., eds.). Academic Press, New York, p. 464.

Thoye R. A. (1971), Effects of halothan, anoxia and haemorhage upon canine whole body skeletal muscle and splanchnic excess lactate production. Anaesthesia **35**, 394–400.

Van Der Vies J. (1954), Two methods for the determination of glycogen in liver. J. Biochem. **57**, 410–416.

Woodward D. O. M. and Woodward V. W. (1978), Concept of Molecular Genetics, TMH Edition. Tata McGraw Hill Publishing Company Ltd., New Delhi.