Indian Journal of Experimental Biology
Vol. 42, April 2004, pp. 413-418

Toxic and sub-lethal effects of oleandrin on biochemical parameters of fresh water air breathing murrel, *Channa punctatus* (Bloch.)

Sudhanshu Tiwari & Ajay Singh*

Natural Product Laboratory, Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur 273009, India

Received 21 February 2003; revised 17 December 2003

Active compound oleandrin extracted from *Nerium indicum* (Lal Kaner) leaf has potent piscicidal activity. The piscicidal activity of oleandrin on freshwater fish *C. punctatus* was both time and dose dependent. Exposure to sub-lethal doses of oleandrin for 24hr and 96hr to fish caused significant alteration in the level of total protein, total free amino acid, nucleic acid, glycogen, pyruvate, lactate and enzyme protease, phosphatases, alanine aminotransferase, aspartate aminotransferase and acetylcholinesterase activity in liver and muscle tissues. The alterations in all the above biochemical parameters were also significantly time and dose dependent. The results show a significant recovery in all the above biochemical parameters, in both liver and muscle tissues of fish after the 7th day of the withdrawal of treatment. Toxicity persistence test of oleandrin on juvenile *Labeo rohita* shows that fish seed of common culturing carp can be released into rearing ponds after three days of oleandrin treatment. It supports the view that the oleandrin is safer and may be useful substitute of other piscicides for removing the unwanted freshwater fishes from aquaculture ponds.

Keywords: Air breathing murrel, *Channa punctatus*, Oleandrin
IPC Code: Int. Cl.[7] A61

Control of wild, resident, carnivorous species of fish e.g. *Channa punctatus, Channa marulius, Channa gachua*, *Wallago attu*, *Clarias* etc. from fish culture ponds prior to stocking is necessary because many species prey upon fingerlings of cultivated species of carps[1, 2]. Air breathing predatory fish species cause special problems because they are carnivorous and survive in moist burrow even when the ponds are drained[1]. Application of synthetic piscicides is one of the methods used to increase aquaculture production. But due to their long term persistence and slow degradability in the water and fish body, they adversely affect the production and quality[3] of fish and contaminate the aquatic environment. To solve this problem, studies have been carried out on the possibility of using local plants as piscicides[2,4]. Because the toxic effect of plant products is degraded easily within 7-12 days[5], they are also safe for users. Several plants belonging to different families, having a number of compounds (saponines, tannins, alkaloids, alkenyl phenols, di- and triterpenoids etc.) with high piscicidal activity are used to control predatory fish, diseases causing insect such as mosquito larvae and harmful freshwater snails[6-9]. The toxicity of crude extract of *Nerium indicum* leaf and stem bark to fresh- water snail *Lymnaea acuminata; Indoplanorbis exustus* and the freshwater air breathing murrels, *Channa punctatus* has been established[4, 10]. In the present investigation, the toxicity and sub-lethal effect of active compound oleandrin extracted from *Nerium indicum* leaf have been described on different biochemical parameters of freshwater air breathing fish, *Channa punctatus* (Bloch.) in the laboratory, because such substances cannot be put to commercial use without a study of these aspect as well.

*Correspondent author
Phone: 0551-2201171, 2202127
E-mail: ajay_s@sancharnet.in

Materials and Methods

Collection of experimental animal—The specimens of *Channa punctatus* (15.7±1.35 cm total length) were collected from Ramgarh lake of Gorakhpur district. The collected fishes were stored in glass aquaria containing 100 l de-chlorinated tap water. Experimental conditions of water were determined by the methods of APHA/WEF[11]. The water analysis indicates atmospheric temperature 30.5°±1.5°C; water temperature 28°±1°C; *p*H 7.3 to 7.5; dissolved oxygen 6.6 to 7.8 mg/l; free carbon dioxide 4.1 to 6.2 mg/l; bicarbonate alkalinity 106.0 to 109.0 mg/l. Prior to experiment, fish were allowed to acclimate to laboratory conditions for 7 days. Diseased, injured and dead fish (if any) were removed to prevent the decomposition of the body. Water was changed every 24 hr.

Collection of plant material—The plant (*Nerium indicum*; Family-Apocynaceae) was collected locally from Botanical garden of Gorakhpur University and identified by Prof. S.K. Singh, Plant Taxonomist, D.D.U. Gorakhpur University, Gorakhpur, U.P., India, where a voucher specimen is deposited.

Isolation of oleandrin—Oleandrin was isolated from *N. indicum* leaf by the method of Yamuchi *et al*[12]. Fresh leaves of *N. indicum* were dried in incubater at 80°C, powdered with electrical device and percolated with methanol. The percolate was concentrated, diluted with water to 50% and filtered. The filtrate was extracted with benzene and crystallized from ethylacetate-hexane to give oleandrin. Identification of compounds was further confirmed with authentic sample of oleandrin ($C_{32}H_{48}O_9$), supplied by Sigma Chemical Co. U.S.A.

Treatment condition for toxicity testing—Toxicity experiment was performed by the method of Singh and Agarwal[13]. The fishes were exposed for 24, 48, 72 or 96 hr at four different concentrations of oleandrin. Six aquaria were used for each dose and each aquarium accommodated ten fishes in six litre de-chlorinated tap water. Control fishes were kept in similar condition without treatment. Mortality was recorded every 24hr up to 96hr exposure period. Fishes were considered dead if they failed to respond to stimulus providing with glass rod. LC values, upper and lower confidence limits, slope value,'t' ratio, 'g' factor and heterogeneity were calculated according to Probit log method by POLO computer programme of Russel *et al*[14].

Persistence of oleandrin toxicity in water—One litre of oleandrin treated water (28.5°±1.0°C) was taken from the stock treated with 24hr LC_{90} (2.83 mg/l) of *C. punctatus* for 6 days. Into this, juvenile *Labeo rohita* (average length of 8.3±1.0 cm) were stocked at 5 fish/l without aeration. The number of dead fish was recorded at every 24hr up to 6 days after immersion. Control juveniles were kept in similar condition without any treatment.

Treatment conditions for biochemical estimation—Adult specimens of *C. punctatus* were kept in glass aquaria containing 6 l de-chlorinated tap water. Each aquarium stored ten experimental fishes. Fishes were exposed for 24 or 96hr to 40 and 80% of 24 or 96hr LC_{50} doses of oleandrin. After completion of treatment the test fishes were removed from aquaria and washed with water and killed by severe blow on head. The fishes were operated and their liver and muscle were quickly dissected out in ice tray and used for biochemical analyses. Control fishes were kept in similar condition without any treatment. Each experiment was replicated at least six times and the values expressed as mean ±SE of 6 replicates. Student '*t*' test was applied to express the level of significance between control and experimental data[15].

Parameters—Protein[16], total-free amino acid[17], nucleic acids (DNA and RNA)[18], glycogen[19, 20], pyruvate[21], lactate[22], proteases[23], acid and alkaline phosphatase[24-26], alanine and aspartate aminotransferase[27] and acetylcholinesterase[28, 29] were estimated.

Recovery experiment—In order to see effect of withdrawal of oleandrin treatment, the fishes were exposed for 96hr to 80% of the LC_{50} (96hr) and one half of the fishes were sacrificed and the activity of all above biochemical parameters were measured in liver and muscle tissues. The other half was transferred to freshwater, which was changed every 24hr for the next 7 days. After 7 days of withdrawal treatment all the above biochemical parameters were analyzed in liver and muscle tissues of fishes. Control fishes were kept in similar condition without any treatment.

Results and Discussion

Behavioural changes—Exposure of oleandrin, caused significant behavioural changes in *C. punctatus*. After treatment, all the experimental fishes immediately settled down at the bottom of aquarium. Within 5-10 min, the breathing of fishes was affected and they came to the water-air interface for air breathing. After 30-60 min, their swimming activity also slowed down, settle down at the bottom of the aquarium and in cluster. After some time the operculum movement of fishes were slowed down although they try to stay at upper water surface. Finally their all activities ceased and at last died.

Toxicity experiment—LC values (LC_{10}, LC_{50} or LC_{90}) of oleandrin for *C. punctatus* for periods ranging from 24 to 96hr are presented in Table 1. The toxicity was both time and dose dependent. There was a significant negative correlation ($r = -0.9987$; $P<0.001$) between LC_{50} values and exposure periods. Thus with an increase in exposure period the LC_{50} values of oleandrin decreased from 2.64 mg/L (24 hr)> 2.51 mg/L (48 hr)> 2.40 mg/L (72 hr) and 2.34 mg/L (96 hr) for *C. punctatus* (Table 1).

Table 2 shows that the mortality of juvenile *L. rohita* decreased with time after oleandrin application, as was zero after three days.

Data on the toxicity of oleandrin indicate behavioural changes in fishes. Increased opercular move-

ment seems to be an index of stress. Subsequent decrease in opercular movement may be due to dominant air breathing and lower gill ventilation to avoid damage to gill epithelium. From the present study, it is also evident that oleandrin caused significant inhibition of AChE enzyme activity in both liver and muscle tissues of *C. punctatus*. Such behavioural anomalies are due to inhibition of enzyme acetylcholinesterase (AChE) responsible for the termination of cholinergic impulse by the hydrolysis of neurotransmitter acetylcholine released during synaptic transmission[30].

Toxicity data demonstrates that the oleandrin is more effective (24hr LC_{50}; 2.64mg/l) in controlling predatory fish *C. punctatus* in compression to other prevalent synthetic carbamates and organophosphates pesticides[31] such as dichlorovas (24hr LC_{50}; 3.15 mg/l); dimathoate (24hr LC_{50}; 17.99 mg/l) and carbaryl (24hr LC_{50}; 18.51mg/l).

The slope values of toxicity data (Table 1) were steep and heterogeneity factor less than 1 indicates the result found to be within the 95% confidence limits of LC values. The regression test ('t' ratio) was greater than 1.96 and the potency estimation test ('g' value) was less than 0.5 at all probability levels.

Biochemical effects—Exposure of fish to sub-lethal doses of 40 and 80% of 24 and 96 hr LC_{50} of oleandrin caused significant alterations in nitrogenous as well as carbohydrate metabolism of the fish *C. punctatus* in both liver and muscle tissues (Table 3).

Protein, nucleic acids (DNA and RNA), glycogen, pyruvate level and activity of enzyme acid, alkaline phosphatase, acetylcholinesterase (AChE) were significantly reduced ($P<0.05$), while free amino acid, lactate level and proteases, alanine aminotransferase (ALAT), aspartate aminotransferase (AAT), enzyme activity were significantly enhanced ($P<0.05$) in liver and muscle tissues after exposure to sub-lethal doses (Table 3).

Seven days withdrawal experiment of 80% of 96hour LC_{50} of oleandrin (Table 3) showed significant ($P<0.05$) recovery in the level of protein, amino acid, nucleic acid, glycogen, pyruvate and lactate and in the activity of enzyme acid phosphatase, alkaline phosphatase, protease, aspartate aminotransferase, alanine aminotransferase and acetylcholinesterase.

After exposure to sub-lethal concentration of oleandrin, fishes came under stress condition. During stress condition, fish needed more energy to detoxify the toxicants and try to minimize the adverse effect of

Table 2—Toxicity persistence of oleandrin in water as mortality of juvenile *Labeo rohita*
[Values are mean ± SE]

Elapsed time (day)	mortality (%)
1	100.0 ± 0.0
2	100.0 ± 0.0
3	90.0 ± 5.0
4	Zero

There was no mortality in control groups.

Table 1—Toxicity (LC_{10}, LC_{50} and LC_{90}) of oleandrin, (glycoside) extracted from *Nerium indicum* leaf against *Channa punctatus* at different time intervals

Exposure periods (hr)	Effective dose (mg/l)	Limits (mg/l)		Slope value	'g' factor	't' ratio	Heterog-eneity
		LCL	UCL				
24	LC_{10}=2.47	2.40	2.51	3.28±1.27	0.06	8.21	0.42
	LC_{50}=2.64	2.61	2.67				
	LC_{90}=2.83	2.78	2.90				
48	LC_{10}=2.38	2.33	2.41	5.18±0.63	0.06	8.32	0.10
	LC_{50}=2.51	2.48	2.54				
	LC_{90}=2.65	2.61	2.70				
72	LC_{10}=2.24	2.18	2.28	4.50±1.51	0.05	9.21	0.80
	LC_{50}=2.40	2.37	2.43				
	LC_{90}=2.58	2.53	2.64				
96	LC_{10}=2.21	2.15	2.25	4.44±1.51	0.05	8.93	0.66
	LC_{50}=2.38	2.34	2.41				
	LC_{90}=2.56	2.51	2.62				

Batches of ten fishes were exposed to four different concentrations of oleandrin.
Concentrations given are the final concentrations (w/v) in aquarium water.
Regression coefficient showed that there was significant ($P<0.05$) negative correlation between exposure time and different LC values.
LCL = Lower confidence limit; UCL = Upper confidence limit
There was no mortality in control groups.

toxic compounds. Since fish have a very little amount of carbohydrates [32], the protein is the next alternative source of energy to meet the increased energy demand. It is mainly involved in the repair of cell which is the chief source of nitrogenous metabolism. The depletion of protein fractions in liver and muscle tissues may be due to their degradation and possible utilization of degraded products for metabolic purposes. Increment of free amino acid level was the result of breakdown of protein for energy requirement and impaired incorporation of amino acids in protein synthesis[5]. The decrease in total protein level and increase in total free amino acid level in both the tissues suggest the high protein hydrolytic activity due to elevation of protease activity. Data obtained in the present investigation indicate inhibitory effect of oleandrin on DNA synthesis, which resulted in the reduction of RNA level. It is possible that the extracted compound inhibited the enzymes necessary for DNA synthesis as suggested by Mahendru[33].

Carbohydrates are the primary and immediate source of energy. In stress conditions, its reserves levels depleted to meet energy demand of the fish. Depletion of glycogen may be due to direct utilization for energy generation, a demand caused by active compound induced hypoxia. Reports are available on the effect of muscular exercise on liver glycogen energy reserves in fish, which get depleted[34]. Liver glycogen levels are depleted during acute hypoxia or physical disturbances in the fish[35]. Finally, glycoge-

Table 3—Changes in various biochemical parameters in liver (L) and muscle (M) tissues of *C. punctatus* after 24hr and 96hr exposure to 40; 80% of 24hr and 96hr LC_{50} of Oleandrin extracted from *N. indicum* leaf and 7th days after withdrawal
[Values are mean ±SE of 6 replicates. Figures in parentheses are % increase (+) or decrease (-) over control]

		Control	24hr		96hr		7th day after withdrawal
			40% LC_{50} (1.06 mg/l)	80% LC_{50} (2.12 mg/l)	40% LC_{50} (0.95 mg/l)	80% LC_{50} (1.90 mg/l)	
TP	L	131.2±1.2	89.7±1.9*(-68)	70.5±1.8*(-54)	80.3±1.6*(-61)	66.4±1.5*(-51)	120.49±0.1†(-92)
	M	150.9±1.5	85.6±1.1*(-57)	72.9±1.3*(-48)	71.6±1.2*(-47)	64.9±1.4*(-43)	140.60±1.5†(-93)
AA	L	9.8±0.4	15.0±0.5*(+154)	23.7±0.6*(+243)	16.2±0.7*(+166)	23.3±0.5*(+239)	11.86±0.7†(+155)
	M	16.9±0.5	20.0±0.8*(+118)	27.4±0.9*(+161)	20.8±0.5*(+123)	29.8±0.6*(+176)	21.96±0.8†(+150)
DA	L	35.9±0.2	30.0±0.3*(-86)	26.4±0.3*(-73)	30.2±0.7*(-84)	23.1±0.6*(-64)	31.21±0.7†(-87)
	M	34.9±0.5	25.5±0.4*(-73)	23.1±0.2*(-66)	23.2±0.3*(-66)	19.5±0.5*(-55)	31.37±0.1†(-90)
RA	L	36.0±1.8	29.0±0.6*(-80)	23.0±0.4*(-63)	27.9±0.3*(-77)	21.2±0.3*(-59)	31.7±0.1†(-88)
	M	37.1±0.4	22.5±0.5*(-61)	19.6±0.5*(-53)	18.9±0.3*(-51)	16.3±0.5*(-44)	31.9±0.8†(-86)
PR	L	0.50±0.04	0.60±0.02*(+119)	0.72±0.02*(+144)	0.64±0.02*(+129)	0.76±0.02*(+152)	0.59±0.02†(+118)
	M	0.34±0.02	0.47±0.02*(+136)	0.63±0.02*(+184)	0.49±0.01*(+143)	0.67±0.02*(+194)	0.44±0.01†(+128)
GL	L	2.04±0.06	1.88±0.04*(-92)	1.39±0.05*(-68)	1.84±0.04*(-90)	1.35±0.04*(-66)	1.63±0.05†(-80)
	M	1.70±0.01	1.43±0.04*(-84)	1.12±0.03*(-66)	1.38±0.07*(-81)	1.03±0.05*(-60)	1.33±0.06†(-78)
PY	L	2.34±0.02	1.75±0.05*(-75)	1.46±0.03*(-62)	1.53±0.03*(-65)	1.40±0.03*(-60)	2.11±0.05†(-90)
	M	1.99±0.02	1.52±0.03*(-78)	1.11±0.03*(-57)	1.34±0.02*(-69)	1.05±0.01*(-54)	1.61±0.08†(-81)
LA	L	1.50±0.03	2.72±0.10*(+181)	4.56±0.12*(+304)	3.29±0.23*(+219)	4.45±0.16*(+297)	1.86±0.04†(+124)
	M	1.28±0.04	4.70±0.09*(+367)	5.25±0.05*(+410)	4.52±0.34*(+353)	5.23±0.22*(+409)	1.54±0.03†(+120)
AC	L	0.14±0.001	0.10±0.001*(-71)	0.09±0.001*(-64)	0.10±0.002*(-70)	0.08±0.001*(-62)	0.13±0.002†(-90)
	M	0.13±0.003	0.14±0.006(-97)	0.13±0.005*(-90)	0.14±0.004*(-94)	0.11±0.001*(-78)	0.12±0.001†(-88)
AK	L	0.49±0.004	0.47±0.005*(-95)	0.40±0.003*(-82)	0.46±0.01*(-94)	0.38±0.005*(-77)	0.42±0.002†(-85)
	M	0.48±0.009	0.43±0.017*(-90)	0.41±0.005*(-85)	0.43±0.02*(-89)	0.37±0.02*(-77)	0.39±0.001†(-81)
AL	L	3.83±0.06	4.37±0.08*(+114)	5.85±0.13*(+153)	5.13±0.02*(+134)	6.47±0.06*(+169)	4.60±0.07†(+120)
	M	3.01±0.09	3.32±0.02*(+110)	5.10±0.24*(+169)	4.16±0.10*(+138)	5.73±0.16*(+190)	3.49±0.06†(+116)
AT	L	1.50±0.03	1.58±0.02*(+105)	2.27±0.08*(+151)	1.93±0.03*(+128)	2.44±0.06*(+162)	1.81±0.05† (+120)
	M	1.32±0.07	1.43±0.10*(+108)	2.20±0.08*(+167)	1.73±0.05*(+131)	2.40±0.13*(+182)	1.61±0.06†(+122)
AE	L	0.19±0.02	0.12±0.004*(-65)	0.07±0.008*(-37)	0.10±0.006*(-53)	0.07±0.006*(-32)	0.17±0.010†(-88)

Data were analysed through Student's *t* test.
*, Significant (P< 0.05), when treated groups were compared with controls.
†, Significant (P< 0.05), when withdrawal groups were compared with treated groups
Protein (TP), Amino acid (AA), DNA (DA), RNA (RA), Pyruvate (PY) and Lactate (LA) were expressed in µg/mg while glycogen (GY), protease (PR), alanine-aminotransferase (AL)/aspartate-aminotransferase (AT), acid (AC)/alkaline (AK) phosphatase and acetylcholinesterase (AE) expressed in mg/g, µmoles tyrosine/mg protein/h, µmoles pyruvate/mg protein/h, ρ-Nitro phenol/30min/mg protein and µmol 'SH'/min/mg protein, respectively.

nolysis seems to be the result of increased secretion of catecholamine due to stress. Pesticides also inhibit energy production by suppressing aerobic oxidation of carbohydrate resulting in energy crisis in animals[36].

The end product of glycolysis under anaerobic condition in tissue is lactic acid, whereas the pyruvate level in tissue can be taken as a measure of aerobic condition of tissue depending on the availability of molecular oxygen. The lactate level of tissues acts as an index of anaerobiosis, which may be beneficial for fish to tolerate hypoxic condition under pesticide stress condition[37]. In liver and muscle both aerobic and anaerobic conditions are likely to operate depending on availability of molecular oxygen and other physiological needs imposed by other factor.

With the increase of lactate content during stress there was a decrease in pyruvate content in all the tissues. The decrease in liver and muscle pyruvate level and increase in lactate content suggest a shift towards anaerobiosis as a consequence of hypoxia, created under pesticides toxic impact leading to respiratory distress[38]. The increase in tissue lactate content may be due to its involvement in osmoregulation. During stress condition there was decrease in osmolarity of internal body media of animal by loss of mono as well as divalent cations which are compensated by the animal with the increase of organic ions like lactate, amino acid etc.[39]. The decrease of pyruvate level may be due to its conversion to lactate or due to its mobilization to form amino acids, lipids, triglycerides and glycogen synthesis in addition to its role as a detoxification factor in ammonia toxicity[40]. Both the aspartate and alanine amino transferase function as a link between carbohydrate and protein metabolism by catalyzing the interconversion of strategic compounds like α-ketoglutarate and alanine to pyruvic acid and glutamic acid respectively[41, 42]. The present study indicates higher aminotranferase activity in liver than muscle tissues of control fishes (Table 3). This suggests that liver tissues to be very efficient in utilizing amino acid for metabolic purposes.

In experimental fishes the aminotransferase activities were highly elevated in both the tissues, confirming the augmentation of stress as a consequence of oleandrin. Stress in general is known to elevate aminotransferase activities[41, 43]. Such a situation takes place due to oleandrin. Here the glycogen, which is ultimate energy source decreases (Table 3) resulting in higher demand for carbohydrate and their precursors to keep the glycolytic and TCA cycles at sustained levels to cope the energy demands during stress condition. Since the amino acid level also increased (Table 3) it is evident that both ALAT and AAT activities are being stepped up to cope with the increasing energy demands. In liver and muscle tissues, ALAT predominates over AAT where the feeding of amino acids into energy cycle is more through alanine-pyruvate pathway representing anaerobic tendency of the tissues.

Vorbrodt[44] has reported that alkaline phosphatase is an important enzyme of animal metabolism, which plays an important role in the transport of metabolites across the membranes. The reduction in protein level may be due to the inhibition of alkaline phosphatase activity, as it plays an important role in protein synthesis[45] and also involved in the synthesis of certain enzymes[46].

It is proposed that oleandrin may be useful substitute for synthetic piscicides in killing predatory and weed fishes from fish and shrimp culture ponds. Its short term persistence in water is also an advantage in its use.

Acknowledgement

Sudhanshu Tiwari is thankful to ICAR, New Delhi for financial support.

References

1 Jhingran V G, *Fish and fisheries of India*, (Hindustan Publishing Corporation, Delhi, India) 1991, 954.

2 Chiayvareesajja S, Rittibhonbhun N, Hongpromyart M & Wiriyachitra P, Toxicity of the thai piscicidal plant, Maesa ramentacea, to freshwater fishes in ponds, *Aquaculture*, 158 (1997) 229.

3 Waliszewski S M, Aguirre A A, Benitz A, Infanzon R M, Infanzon R, Rivera J, Organochlorine pesticides residues in Human Blood Serum of inhabitants of Veracruz, Mexico. *Bulletin of Environmental Contamination and Toxicology*, 62 (1999) 397.

4 Singh D & Singh A, The acute toxicity of plant origin pesticides into the fresh water fish *Channa punctatus*, *Acta Hydrochim Hydrobiol*, 28 (2000) 92.

5 Chakroff M, *Fresh water fish pond culture and management* (A joint peace corps and volunteers in Technial Assistance Publication, MD) 1976.

6 Singh A, Singh D K, Mishra T N & Agarwal R A, Molluscicides of plant origin, *Biol Agric Hortic*, 13 (1996) 205.

7 Singh K, Singh A & Singh D K, The use of piperonyl butoxide and MGK-264 to improve the efficacy of plant derived molluscicides, *Pest Sci*, 54 (1998) 145.

8 Singh K, Singh A & Singh D K, Synergistic effect of MGK-264 and piperonyl butoxide on the toxicity of plant molluscicides, *Chemosphere*, 36 (1998) 3055.

9 Tiwari S & Singh A, Piscicidal activity of active compound of *Euphorbia royleana* latex extracted through different organic solvents, in *Proceeding of first national interactive*

meet on medicinal and aromatic plants, edited by A K Mathur, S Dwivedi, D D Patra, G D Bagchi, N S Sangwan, A Sharma & S P S Khanuja (CIMAP, Lucknow, India) 2003, 330.

10 Singh D K, Singh A & Agarwal R A, *Nerium indicum* as a potent molluscicide of plant origin, *J Med Appl Malacol*, 5 (1993) 93.

11 APHA/AWWA/WEF, *Standard methods for the examination of water and wastewater, 20th edition*, (American public health association, New York, USA) 1998.

12 Yamuchi T, Mori Y & Ogata Y, Δ^{16}-Dehydroadynerigenin Glycosides of *Nerium odorum*, *Phytochemistry*, 12 (1973) 2737.

13 Singh A & Agarwal R A, Possibility of using latex of euphorbiales for snail control, *Sci Tot Environ*, 77 (1988) 231.

14 Russel R M, Robertson J L & Savin N E, POLO A new computer programme for probit analysis, *Bull Entomol Soc Am*, 23 (1977) 209.

15 Sokal R R & Rohlf F J, *Introduction of biostatistics* (W.H. Freeman and Company, San Francisco) 1973, 368.

16 Lowry O H, Rosenbrough N J, Farr A L & Randall R J, Protein measurement with foline phenol reagent, *J Biol Chem*, 193 (1951) 265.

17 Spices J R, Colorimetric procedures for amino acids, in *Methods of enzymology*, edited by S P Calowick & N O Kaplon (Academic Press, New York) 1957, 468.

18 Schneider W C, Determination of nucleic acids in tissue by pantose analysis, in *Methods of enzymology*, edited by S P Calowick & N O Kaplon (Academic Press, New York) 1957, 680.

19 Van der Vies J, Two methods for determination of glycogen in liver, *Biochem J*, 57 (1954) 410.

20 Mahendru V K & Agarwal R A, Changes in metabolism in various organs of the snail *Lymnaea acuminata* following exposure to trichlorfon, *Acta Pharmacol Toxicol*, 48 (1982) 377.

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

22 Huckabee W E, in *Hawk's physiological chemistry*, 14th edition edited, by L B Oser (Tata Mc Graw-Hill, New Delhi) 1961, 1103.

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

24 Anderson M A & Szcypinski A J, *Am J Clin Path*, 17 (1947) 571, from *Methods of enzymatic analysis*, edited by U H Bergmeyer (Academic Press, New York) 1967.

25 Bergmeyer U H, *Methods of enzymatic analysis* (Academic Press, New York) 1967, 1129.

26 Singh R & Agarwal R A, Chemosterilization and its reversal in the snail *Lymnaea acuminata*, *Acta Pharmacol et toxicol*, 52 (1983) 112.

27 Reitman S & Frankel S, A colorimetric method for the determination of glutamic-oxaloacetic and glutamic-pyruvic transminases, *Am J Clin Pathol*, 33 (1957) 1.

28 Ellman G L, Courtney K D, Andres Jr V & Featherstone R M, A new and rapid colorimetric determination of acetylcholinesterase activity, *Biol Pharmacol*, 7 (1961) 88.

29 Singh D K & Agarwal R A, Comparative study of cholinesterase in two snails *Pila globosa* and *Lymnaea acuminata*, *J Physiol*, 78 (1982) 467.

30 Gill T.S, Pandey J & Tewari H, Individual and combined toxicity of common pesticides to teleost, *Puntius conchonius*, Hamilton, *Indian J Exp Biol*, 29 (1991) 145.

31 Srivastava V K & Singh A, Study of seasonal variation in toxicity of frequently used commercial organophosphates, carbamate and synthetic pyrethroids pesticides against fresh water fish *Channa punctatus* and behavioural responses of treated fish, *Malay App Biol*, 30 (2001) 17.

32 Sambasiva Rao K R S, *Pesticide impact on fish metabolism*. (Discovery Publishing House, New Delhi, India) 1999.

33 Mahendru V K, *Studies on pharmacology of molluscicides on the gastropod Lymnaea acuminata*, Ph.D. thesis, Gorakhpur University, Gorakhpur India, 1981.

34 Nath K & Kumar K, Toxic impact of hexavalent chromium on the blood pyruvate of a teleost *Colisa fasciatus*, *Acta Hydrochem et Hydrobiol*, 5 (1987) 531.

35 Heath, A G & Fritechard A W, Effect of severe hypoxia on carbohydrate energy. Stores and metabolism in two species of fresh water fish, *Physiol Zool*, 38 (1965) 325.

36 Kohli K K, Sharma S C, Bhatia S C & Venkita Subramonian T A, Biochemical effect of chlorinate insecticides DDT and dieldrin, *J Sci Ind Res*, 34 (1975) 462.

37 Thoye R A, Effect of halothan, anoxia and hemorrhage upon canine whole body skeletal muscle and splanchine excess lactate production, *Anaesthesiology*, 35 (1971) 394.

38 Siva Prasad Rao K, *Studies on some aspects of metabolic changes with emphasis on carbohydrate utility in the cell free system of the teleost, Tilapia mossambica (Peters) under methyl parathion exposure*, Ph.D. Thesis, S.V. University, Tirupti, India, 1980.

39 Kaber Ahammad Sahib I, Siva Prasad Rao K, Sambasiva Rao K R S & Ramana Rao K V, Sub-lethal toxicity of malathion on the protease and free amino acid composition in the liver of the teleost, *Tilapia mossambica* (Peters), *Toxicol lett*, 20 (1984) 59.

40 Sathya Prasad K, *Studies on the toxic impact of lindane on tissue metabolic profiles in the fresh water fish, Tilapia mossambica (Peters) with emphasis on carbohydrate metabolism*, Ph. D. Thesis, S V University, Tirupti, India, 1983.

41 Knox W E & Greengard O, in *An introduction to enzyme physiology*, Advan. Enzyme Regul. Edited by G Weber (Pergamon Press, New York, London) Volume 3, 1965, 247.

42 Martin D W, Mayers P A & Rodwell V W, *Harper's review of biochemistry* (Lange Medical Publications, Maruzen, Asia) 1983.

43 Natarajan G M, Inhibition of branchial enzymes in snake head fish (*Channa striatus*) by oxy demetom-methyl, *Pest Biochem Physiol*, 23 (1985) 41.

44 Vorbrodt A, The role of phosphatase in intracellular metabolism, *Postepy Hig Med Dosw*, 13 (1959) 200.

45 Pilo B, Asnani M V & Shah R V, Studies on wound healing and repair in pigeon liver: II. Histochemical studies on acid and alkaline phosphatase during the process, *J Anim Morphol Physiol*, 19 (1972) 205.

46 Sumner A T, The cytology and histology of the digestive gland cells of *Helix*, *Q J microsc Sci*, 106 (1965) 173.