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Metabolic Changes in The Snake Head Fish *Channa punctatus* Due to Latices of *Euphorbia royleana*

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Abstract

Laboratory evaluation was made to assess the short as well as long term effects of methanolic extract of *Euphorbia royleana* latex against nontarget freshwater fish *Channa punctatus*, which shares the freshwater habitat with target animals. Exposure of sublethal doses (4.70 mg·l; 9.41 mg·l for 24 h and 3.67 mg·l and 7.34 mg·l for 96 h) of methanolic extract caused significant ($P<0.05$) time and dose dependent reduction in the levels of total protein, nucleic acids (DNA and RNA), glycogen, pyruvate and significant ($P<0.05$) enhancement in the levels of total free amino acid, lactate and activity of enzyme protease in both liver and muscle tissues of freshwater fish *C. punctatus*. Withdrawal study also shows that there is a significant recovery in all the above biochemical parameters, in both tissues of fish after the 7^{th} day of the withdrawal of treatment, which supports the view that the plant product is safe to be used as pesticides for control of freshwater target organisms as well as predatory and weed fishes from freshwater ponds.

Introduction

In recent years, use of medicinal plants as effective alternatives to synthetic pesticides and fertilizers has gained more importance because they are more effective, less expensive, biodegradable and safe for mankind and environment, than synthetic pesticides (Marston and Hostettmann 1985; Singh et al. 1996).

Several plants belonging to different families, which posses a number of compounds as saponins, tannins, alkaloids, di- and tri-terpenoids etc. have high pesticidal activity and used in freshwater bodies to control harmful snails, disease causing insects, such as mosquito larvae and weed fishes (Hostettmann and Lea 1987; Okunji and Iwu 1988; Gopalsamy et al. 1990; Alard et al. 1991 and Singh et al. 1996, 1998 a, b).

Despite the above-mentioned advantages, we are interested in the ecotoxic properties of plant origin pesticides, since plant origin pesticides cannot be used directly in freshwater unless their toxicity and sublethal long-term effects have been studied on nontarget animals, sharing the habitat with target animals.

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Euphorbia royleana is a common medicinal plant in India, having high molluscicidal properties (Singh and Agarwal 1984a, b, 1988, 1990). The latex of this plant is anthelmintic, cathartic and has been used for a variety of conditions such as snakebite, obstipation and skin disease (Chopra et al. 1958).

The toxicity of methanol, chloroform, acetone and diethyl ether extracts of *E. royleana* latex against freshwater air breathing fish *C. punctaus* were already established (Tiwari and Singh 2002). The rank in the order of toxicity was methanol 11.76 mg·l (24 h LC_{50}) > acetone 12.88 mg·l (24 h LC_{50}) > chloroform 13.34 mg·l (24 h LC_{50}) and > diethyl ether 16.19 mg·l (24 h LC_{50}). Thus methanolic extract of *E. royleana* latex has maximum piscicidal activity against *C. punctaus* (Tiwari and Singh 2002).

The present investigation deals with the biochemical effect of sublethal doses of methanol extract of *E. royleana* latex on the levels of total protein, total free amino acid, nucleic acids (DNA and RNA), glycogen, pyruvate, lactate and activity of enzyme protease, in the liver and muscle tissues of *C. punctatus*, an important fish of Indian capture fishery.

Materials and Methods

Collection of experimental animals

C. punctatus (Bloch.) (15.7±1.35 cm) collected from Ramgarh Lake in the Gorakhpur district of U.P. India were stored in glass aquaria containing 100 l of dechlorinated tap water. Experimental conditions of water (atmospheric temperature 30.5±1.5°C; water temperature 28.0±1.0°C; pH 7.2 to 7.4; dissolved oxygen 6.8 to 7.5 mg·l; free carbon dioxide 4.3 to 6.2 mg·l; and bicarbonate alkalinity 106.0 to 109.0 mg·l) were determined following the methods of APHA/WPCF (1998).

Prior to the experiment, the fish were allowed to acclimate to laboratory conditions for seven days. Diseased, injured and dead fish (if any) were removed as soon as possible to prevent the decomposition of the body. Water was changed every 24 h. Average sized (13.4±1.26 cm) adult animals were used for the experiment.

Collection of plant materials

The plant *E. royleana* (Euphorbiaceae) was collected from the botanical garden of D.D.U. Gorakhpur University, Gorakhpur and identified by Prof. S.K. Singh, Plant taxonomist, Department of Botany, DDU Gorakhpur University, Gorakhpur, U.P., India, where a voucher specimen is deposited.

Preparation of methanolic extracts of latex (MEL)

The white, milky latex of *E. royleana* was drained into glass tubes by cutting the stem apices. This latex was lyophilized at –40°C and lyophilized powder was stored for further use. The wet weight of one ml latex of *E.*

royleana was 1.370 gm and dry weight (lyophilized at –40°C) was 0.530 gm. One gm freeze-dried latex powder was mixed with 100 ml of methanol organic solvent. The whole solution was left for one hour at room temperature and then centrifuged at 5000 g for 25 min. Solvents were removed using a vacuum pump. Thus, methanol extract of *E. royleana* latex, which was used for further study was obtained in dried powdered form (0.540 gm).

C. punctatus were treated with MEL according to the method of Singh and Agarwal (1988). Ten fish were kept in each glass aquaria containing 6 l dechlorinated tap water. Fish were exposed for 24 h to 4.70 mg·l and 9.41 mg·l (40% and 80% for 24 h LC_{50}) and 3.67 mg·l to 7.34 mg·l for 96 h (40% and 80% for 96 h LC_{50}) of the MEL. Control animals were held in similar condition without any treatment. After completion of the exposure period, fish were removed from the aquaria, washed with water and killed instantly. The liver and muscle from the left side of the dorsal fin were quickly dissected out and used for biochemical analysis. Each of the above experiments was replicated at least six times and the values were expressed as mean ±SE of six replicates. Student test was applied to locate significant changes with controls (Sokal and Rohlf 1973).

Biochemical analysis

Protein levels were estimated following the method of Lowry et al. (1951) using bovine serum albumin as standard. Homogenates (5 mg·ml, w/v) were prepared in 10% TCA. Values have been expressed as µg protein·mg of tissue. Total free amino acids were estimated using the method of Spices (1957). Homogenates (10 mg·ml, w/v) were prepared in 95% ethanol, centrifuged at 6000 xg and supernatant was used for amino acid estimation. Standard curves using the same procedure were drawn with known amounts of glycine. Free amino acids have been expressed as µg·mg of tissue. Estimation of DNA and RNA was performed, following the methods of Schneider (1957) using diphenylamine and Orcinol reagents, respectively. Homogenates (10 mg·ml, w/v) were prepared in 5% TCA at 90^0C, centrifuged at 5000 g for 20 min and supernatant was used for estimation. Both DNA and RNA have been expressed as mg·mg tissue. Protease activity was estimated using the method of Moore and Stein (1954). Homogenate (50 mg·ml, w/v) was prepared in cold distilled water. Optical density was measured at 570 nm. Tyrosine solution was taken as standard. The enzyme activity was expressed in m moles of tyrosine equivalent·mg protein·hour. Glycogen was estimated following the Anthrone method of Van der Vies (1954) as modified by Mahendru and Agarwal (1982) for snails. Homogenate (10 mg·ml, w/v) was prepared in cold 5% TCA. The homogenates were filtered and 1.0 ml of filtrate was used for assay. The optical density was compared with a set of glucose standard of varying concentrations. Result has been expressed as mg glycogen·g tissue. Pyruvate level was measured using the methods of Friedemann and Haugen (1943). Homogenate (50 mg·ml, w/v) was prepared in 10% TCA. Sodium pyruvate was taken as standard. Result has been expressed as µg pyruvate·mg tissue. Lactate was estimated in the manner of Huckabee (1961). Homogenate (50 mg·ml, w/v) was prepared in 10%

cold TCA. Sodium lactate was taken as standard. Result has been expressed as µg lactate·mg tissue.

Withdrawal experiment

In order to see the effect of withdrawal treatment, the fishes were exposed for 96 h to 7.34 mg·l. Half of the animals were sacrificed and the activities of all the above biochemical parameters were measured in the liver and muscle tissues of fishes. The other half were transferred to freshwater, which was changed every 24 h for the next seven days. Control animals were held in similar condition without any treatment. Following this, all the above biochemical parameters were measured in the liver and muscle tissues.

Results

Exposure to sublethal doses 4.70 mg·l and 9.41mg·l for 24 h or 3.67 mg·l and 7.34 mg·l for 96 h of MEL, caused significant alterations in nitrogenous as well as carbohydrate metabolism of the fish C. *punctatus* in both liver and muscle tissues (Tables 1 and 2). Protein and nucleic acid (DNA and RNA) levels were significantly reduced ($P<0.05$), while free amino acid level and protease activity were significantly enhanced ($P<0.05$) in liver and muscle tissues after exposure to sublethal doses. Total protein levels were reduced to 21 and 28% of controls after treatment with 7.34 mg·l for 96 h of MEL in liver and muscle, respectively. In addition, DNA level was reduced to 46 and 30% of controls, RNA level was reduced to 33 and 38% of controls, total free amino acid levels were induced to 326 and 200% of controls and protease activity was increased to 143 and 157% of controls after treatment with 7.34 mg·l for 96 h of MEL in liver and muscle, respectively (Table 2).

Glycogen and pyruvate levels were significantly reduced while lactate level was significantly enhanced in liver and muscle tissues after exposure to sublethal doses of *E. royleana* latex extract. Glycogen level was reduced to 66 and 35%, pyruvate level was reduced to 45 and 55% and lactate levels were increased to 178 and 136% of controls after treatment with 7.34 mg·l for 96 h of MEL in the liver and muscle tissues of fish, respectively (Table 2).

Table 2 also shows that on the 7^{th} day after termination of treatment with MEL, there was nearly complete recovery in the levels of protein, amino acid, nucleic acids, glycogen, pyruvate, lactate and activity of enzyme protease.

Discussion

Behavioral response of fish exposed to sublethal concentrations of the compound (s) present in MEL showed that after exposure, fishes were stressed. During stress, fish need more energy to detoxify toxicants and to overcome stress. Since fish have very little carbohydrate, protein is used to meet the increased energy demand. Proteins are mainly involved in the architecture of

Table 1. Changes in total protein, total free amino acids, nucleic acids (DNA and RNA), protease enzyme, glycogen, pyruvate and lactate levels in different tissues of fish *C. punctatus* after exposure to 40 and 80% of LC_{50} (24h) of methanolic extract of *E. royleana* latex.

Parameters	Tissue	Control	40% LC_{50}(24h) (4.70 mg/L)	80% LC_{50}(24h) (9.41 mg/L)
Protein (µg·mg)	Liver	131.24±1.29 (100)	50.18±1.12* (38)	44.64±0.58* (34)
	Muscle	150.89±1.50 (100)	56.0±0.83* (37)	52.02±1.84* (34)
Amino acid (µg·mg)	Liver	7.65±0.48 (100)	13.45±0.55* (185)	17.41±0.64* (227)
	Muscle	14.64±1.02 (100)	18.09±0.29* (123)	20.18±0.97* (137)
DNA (mg·mg)	Liver	35.87±0.24 (100)	24.38±0.38* (68)	20.77±0.32* (57)
	Muscle	34.85±0.52 (100)	12.10±0.58* (34)	11.50±0.07* (32)
RNA (mg·mg)	Liver	36.01±1.83 (100)	13.68±0.26* (38)	12.29±0.06* (34)
	Muscle	37.08±0.35 (100)	17.22±0.15* (45)	16.07±0.27* (43)
Protease (m tyrosine·mg protein·h)	Liver	0.50±0.041 (100)	0.67±0.021* (134)	0.79±0.024* (159)
	Muscle	0.34±0.017 (100)	0.49±0.020* (144)	0.62±0.014* (182)
Glycogen (mg·g)	Liver	2.04±0.06 (100)	1.45±0.08* (71)	1.178±0.039* (57)
	Muscle	1.70±0.01 (100)	1.01±0.071* (58)	0.67±0.058* (39)
Pyruvate (µg·mg)	Liver	2.34±0.02 (100)	1.15±0.09* (49)	0.90±0.06* (38)
	Muscle	1.99±0.02 (100)	1.18±0.049* (61)	0.90±0.027* (46)
Lactate (µg·mg)	Liver	1.50±0.03 (100)	2.14±0.080* (143)	3.06±0.126* (204)
	Muscle	1.28±0.04 (100)	1.42±0.048* (111)	1.80±0.085* (141)

Values are mean ± SE of six replicates.
Values in parentheses are % of control value.
Data were analyzed through student's test.
*, Significant ($P < 0.05$), when treated groups were compared with controls.

the cell, which is the chief source of nitrogenous metabolism. During chronic periods of stress, they are also a source of energy. Thus the depletion of protein fraction in liver and muscle tissues may have been due to their degradation and possible utilization for metabolic purposes. Increases in free amino acid levels were the result of breakdown of protein for energy and impaired incorporation of amino acids in protein synthesis (Singh et al 1996). It is also attributed to lesser use of amino acids (Seshagiri Rao et al. 1987) and their involvement in the maintenance of an acid-base balance (Moorthy et al. 1984). Natarajan (1985) suggested that stress conditions induce elevation in the transamination pathway. The decreases in total protein level and increases in total free amino acid level in both tissues suggest the high protein hydrolytic activity due to elevation of protease activity. Inhibition of DNA synthesis, thus, might affect both protein as well as amino acid levels by decreasing the level

Table 2. Changes in total protein, total free amino acids, nucleic acids (DNA and RNA), protease enzyme, glycogen, pyruvate and lactate levels in different tissues of fish *C. punctatus* after exposure to 40 and 80% of LC_{50} (96h) of methanolic extract of *E. royleana* latex.

Parameters	Tissue	Control	40% LC_{50} (96h) (3.67 mg/L)	80% LC_{50} (96h) (7.34 mg/L)	7^{th} days after withdrawal
Protein (µg·mg)	Liver	131.24±1.290 (100)	30.28±1.60* (22)	28.29±0.82* (21)	124.25±1.76^{+} (95)
	Muscle	150.89±1.50 (100)	46.91±3.04* (30)	42.93±2.34* (28)	136.82±0.88^{+} (91)
Amino acid (µg·mg)	Liver	7.65±0.48 (100)	19.34±0.37* (252)	24.95±1.50* (326)	10.28±0.88^{+} (134)
	Muscle	14.64±1.02 (100)	24.79±0.70* (171)	28.09±0.54* (200)	20.57±1.98^{+} (141)
DNA (mg·mg)	Liver	35.87±0.24 (100)	18.54±0.35* (51)	16.85±0.13* (46)	33.34±0.45^{+} (93)
	Muscle	34.85±0.52 (100)	11.00±0.14* (32)	10.53±0.16* (30)	31.93±0.40^{+} (92)
RNA (mg·mg)	Liver	36.01±1.83 (100)	13.15±0.27* (36)	12.08±0.15* (33)	33.40±0.60^{+} (93)
	Muscle	37.08±0.35 (100)	16.15±0.13* (43)	14.27±0.10* (38)	34.09±0.49^{+} (92)
Protease (m tyrosine·mg protein·h)	Liver	0.50±0.041 (100)	0.63±0.016* (127)	0.7159±0.017* (143)	0.5562±0.015^{+} (111)
	Muscle	0.34±0.017 (100)	0.48±0.023* (138)	0.54±0.016* (157)	0.39±0.023^{+} (113)
Glycogen (mg·g)	Liver	2.04±0.06 (100)	1.37±0.56* (67)	1.35±0.10* (66)	1.84±0.02^{+} (90)
	Muscle	1.70±0.01 (100)	0.64±0.01* (37)	0.61±0.04* (35)	1.43±0.02^{+} (84)
Pyruvate (µg·mg)	Liver	2.34±0.02 (100)	1.21±0.07* (52)	1.05±0.039* (45)	2.16±0.016^{+} (92)
	Muscle	1.99±0.02 (100)	1.43±0.039* (73)	1.06±0.038* (55)	1.69±0.02^{+} (85)
Lactate (µg·mg)	Liver	1.50±0.03 (100)	2.09±0.045* (139)	2.67±0.109* (178)	1.74±0.024^{+} (116)
	Muscle	1.28±0.04 (100)	1.41±0.027* (110)	1.74±0.089* (136)	1.65±0.047^{+} (129)

Details are as given in table 1.
$^{+}$, Significant ($P< 0.05$), when withdrawal groups were compared with treated groups.

of RNA in protein synthesis machinery. The results of this study suggest that the extracted compound (s) is a potent inhibitor of DNA synthesis, which in turn results in the reduction of RNA level. Mahendru (1981) suggested that anti-AChE compounds attack many enzymes responsible for normal metabolic pathway. Thus, it is possible that extracted compounds might have inhibited the enzymes necessary for DNA synthesis.

Carbohydrates are the primary and immediate sources of energy. In stress condition, carbohydrate reserve is depleted to meet energy demand. Depletion of glycogen may be due to direct utilization for energy generation, a demand caused by active compound induced hypoxia. Several reports are available on the effect of muscular exercise on liver glycogen energy reserves in fish, which get depleted (Black et al. 1962; Nath and Kumar 1987). Liver glycogen levels are depleted during acute hypoxia or physical disturbances in fish (Heath and

Fritechard 1965). Finally, glycogenolysis seems to be the result of increased secretion of catecholamine due to stress. Pesticides are also inhibited energy production by suppressing aerobic oxidation of carbohydrate leading to energy crisis in animals (Kohli et al.1975).

Carbohydrate metabolism is broadly divided into two segments- (1) Anaerobic segment or glycolysis in which break down of glucose or glycogen through Embden Meyerhaf pathway occurs (2) Aerobic segment, which consists of oxidation of pyruvate to acetyl co-A to be utilized through citric acid cycle.

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 level of tissue lactate content acts as an index of anaerobiosis, which might be beneficial for animals to tolerate hypoxic condition (Thoye 1971) under pesticide exposure condition. In the case of 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 factors.

Increases of lactate content were accompanied by a decrease in pyruvate content in all tissues. The decrease in liver and muscle pyruvate levels and increase in lactate content suggest a shift towards anaerobiosis as a consequence of hypoxia, created under pesticides toxic impact leading to respiratory distress (Domsche et al. 1971 and Siva prasad Rao 1980). The increase in tissue lactate content may be due to its involvement in osmoregulation. During stress condition there was a decrease in osmolarity of internal body media of animal by loss of mono as well as divalent cations, which is compensated by the animal with an increase in organic ions like lactate, amino acid etc. (Kaber Ahammad Sahib et al.1984). The decrease in 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 (Sathya Prasad 1983).

We therefore believe that the active compound (s) present in methanolic extract of *E. royleana* latex may eventually be of great value for the control of aquatic target organisms as well as predatory and weed fishes.

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