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Research Paper

Changes in some biochemical parameters in the liver and muscle of *Colisa fasciatus* due to toxicity of ethanolic extract of *Nerium indicum* Mill. (*Lal Kaner*) latex

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Abstract

Present study deals with piscicidal, toxicological and biochemical effects of ethanolic extract of ***Nerium indicum* Mill.** (*Lal Kaner*) latex against freshwater weed fish *Colisa fasciatus*. There was a significant ($P<0.05$) negative correlation between LC values and exposure periods i.e. LC_{50} values decreased from 14.05mg/l (24h) to 5.52mg/l (96h). Sub-lethal exposure of ethanolic latex extract for 24h and 96h caused significant ($P<0.05$) time and dose dependent alterations in the levels of total protein, total free amino acid, nucleic acid, glycogen, pyruvate, lactate and also in the activity of enzyme protease, alanine aminotransferase, aspartate aminotransferase, acetylcholinesterase, lactic dehydrogenase, succinic dehydrogenase and cytochrome oxidase in liver and muscle tissues of fish. Withdrawal experiments shows, their biochemical effects are reversible in action. Thus, ***N. indicum*** latex extract mainly suppress energy production and shifts fish respiration towards the anaerobic segment.

Keywords: Biochemical changes, *Colisa fasciatus*, Freshwater weed fish, *Lal Kaner*, Latex, *Nerium indicum*, Piscicidal activity.

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Introduction

Due to faster growth rate weed fish *Colisa fasciatus* share and better utilize cultured carp habitats and their food and thus adversely affect aquaculture production as well as fish farmer's economy[1]. For eliminating these unwanted fish population from culture ponds, fish farmer made several efforts in which use of plant origin piscicides is effective, eco-friendly and new tool[2-4]. At present, a large number of plant products are commonly used for controlling these unwanted fish population, such as the powdered seed of ***Croton tiglium* Linn.** and ***Barringtonia acutangula* (Linn.) Gaertn.**, derris root powder, tea-seed cake and mahua oil cake[5-7]. The use of derris root powder and tea seed cake is very limited as derris root powder is not readily available and expensive[8], the tea seed cake is also expensive (in 1995 at Hat yai market was US$ 1.4/Kg).

Nerium indicum* Mill.** (Family — Apocynaceae) commonly known as *Lal Kaner* is a common medicinal plant of India useful in the treatment of inflammation of gums, dysentery, bronchitis, asthma, menorrhagia, etc[9]. The toxicity of aqueous latex extract to the freshwater snails, *Lymnaea acuminata* and *Indoplanorbis excustus* has been established earlier[10]. But there is no literature available for toxic effect of ethanolic latex extract on weed fish *Colisa fasciatus*. So in the present study, toxicological and biochemical effects of ethanolic extracts of ***N. indicum latex on freshwater weed fish *C. fasciatus* were investigated.

Materials and Methods

Collection of plant

N. indicum was collected locally and identified by Prof. S.K. Singh, Plant Taxonomist, Department of Botany, D.D.U. Gorakhpur University, Gorakhpur.

Preparation of ethanolic latex extracts

The yellowish milky latex was drained into glass tubes by cutting the stem apices. Latex (10ml) was centrifuged at 1000xg for 20 minutes to remove the resin, than lyophilized at -40°C. The wet weight of one ml latex was 1.06 g and dry weight (lyophilized at -40°C) was 0.160 g. One gram freeze-dried latex powder was mixed with 100 ml of ethanol, after one hour centrifuged at 5000xg for 25 minutes. The filtrate was evaporated to dryness (0.460 g) with the help of vacuum pump and dried, powdered ethanolic latex extract (NL_{EtOH}) was stored in airtight desiccators for further experiments.

Collection of experimental animal

Colisa fasciatus (6.0± 2.5 cm in total length, 7.5± 1.5 g in weight) were collected from Gorakhnath Government Hatchery Center, Chapia, Gorakhpur. The

collected fishes were maintained in glass aquaria containing 100 litres de-chlorinated tap water for seven days to acclimatize under laboratory conditions. The aquarium water was aerated continuously and food was provided in the form of dried, small pellets of Tokyo, special fish food, produced in Japan. The water in aquaria was aerated continuously and changed at every 24 h interval. Experimental water quality parameters were: water temperature 29.5±1.5°C; *p*H 7.2±0.3; dissolved oxygen 7.5±1.0 mg/l; free carbon dioxide 4.4±1.5 mg/l; and bicarbonate alkalinity 105.0±2.0 mg/l. Experimental conditions of water were determined by the method of APHA/WEF[11].

Toxicological experiments

Toxicity experiment was performed by the method of Singh and Agarwal[12], 10 fishes were exposed to four different concentrations of NL_{EtOH} (ethanolic latex extract) in glass aquaria containing six litre of de-chlorinated tap water. Six aquaria were set up for each concentration. Control animals were reared in similar condition without treatment. Mortality was recorded at every 24h up to 96h exposure periods. Fishes were considered dead if they failed to respond to stimulus provided with glass rod. Lethal concentration (LC values), upper and lower confidence limits, slope value,'t' ratio, 'g' factor and heterogeneity were calculated through probit log analysis method by using POLO computer programme of Russel *et al*[13].

Biochemical experiments

The acclimatized fishes were exposed to 5.80 and 11.60 mg/l concentrations of NL_{EtOH} for 24h and 2.21 and 4.42 mg/l concentrations of NL_{EtOH} for 96h exposure period. After completion of treatment, fishes were removed from the aquaria and washed with water. The liver and muscle tissues were excised and total protein[14], total free amino acid[15], Nucleic acid[16] (DNA, RNA), glycogen[17], pyruvate[18], lactate[19] level and activity of enzyme protease[20], alanine (ALAT) and aspartate (AAT) aminotransferase[21], acetylcholinesterase (AChE)[22], lactic dehyrogenase (LDH)[23], succinic dehydrogenase (SDH)[24] and cytochrome oxidase (CyO)[25] were measured.

Withdrawal experiment

In order to see effect of withdrawal of treatment, the fishes were exposed to 4.42 mg/l concentrations of NL_{EtOH} for 96h exposure period and the one half of the fishes were sacrificed and all the above biochemical parameters were measured. The other half was transferred to toxicant free freshwater, which was changed every 24h for the next seven days. Seven days after withdrawal of treatment, all the above biochemical parameters were again measured in both liver and muscle tissues. Control animals were held in similar conditions without any treatment. Each experiment was replicated at least six times and the values are expressed as mean ± SE of six replicates. Student's 't' test and analysis of variance were applied to locate significant changes[26].

Results

Behavioural changes

Exposure of NL_{EtOH}, caused significant visible behavioural changes in *C. fasciatus*. After 60 min, their swimming activity was slow down, feel suffocation, they try to stay at upper water surface for gasping the air, irregular, jerky movements, loss of body equilibrium was pronounced. Finally their all activity decreases and they settle down at the base of water aquaria, formed clusters and died. Fishes of control group was free from such behavioural changes.

Toxicological effects

Data given in Table 1 clearly indicates that fish mortality was both times as well as dose dependent and there was a significant ($P<0.05$) negative correlation between effective doses and exposure periods. Thus, with the increase in exposure period, LC_{50} values decreased from 14.05 mg/l (24h) to 5.52 mg/l (96h). Similar trend was also observed in case of LC_{10} and LC_{90} values (Table 1).

The slope values of toxicity data (Table 1), was steep and heterogeneity factor less than 1. 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 changes

Exposure to sub-lethal doses of NL_{EtOH} of 5.80 mg/l and 11.60 mg/l for 24h (Table 2) and 2.21 mg/l and 4.42 mg/l for 96h (Fig. 1) exposure period caused significant ($P<0.05$) reduction in total protein, nucleic acids (DNA and RNA), glycogen, pyruvate level, enzyme AChE, LDH, SDH, CyO activities and also caused significant ($P<0.05$) enhancement in total free amino acid, lactate level, enzyme proteases, ALAT, AAT activity in both liver and muscle tissues of *C. fasciatus* fish. Seven days withdrawal experiment showed significant ($P<0.05$) recovery in all the above biochemical parameters in both the tissues of fish (Fig. 1).

Table 1: Effective concentrations (LC values) of ethanolic extract of *Nerium indicum* latex against freshwater weed fish *Colisa fasciatus* at different time intervals

Exposure period (hours)	Effective dose (mg/l)	Slope Value	'g' Factor	't' ratio	Heterogeneity
24	LC_{10}=4.89 LC_{50}=14.05 LC_{90}=40.42	2.79±0.61	0.18	4.62	0.14
48	LC_{10}=3.75 LC_{50}=11.04 LC_{90}=32.48	2.74±0.58	0.17	4.74	0.30
72	LC_{10}=3.50 LC_{50}=6.54	4.73±0.70	0.09	6.72	0.38
96	LC_{90}=12.20 LC_{10}=3.19 LC_{50}=5.52 LC_{90}=9.57	5.37±0.91	0.11	5.91	0.52

*There was no mortality in control groups. Batches of ten fishes were exposed to four different concentrations of ethanolic extract of *Nerium indicum* latex. Concentrations given are the final concentrations (w/v) in aquarium water.

Table 2 : Biochemical changes in liver (L) and muscle (M) tissues of *Colisa fasciatus* fish after exposure to 40% and 80% of LC_{50} of ethanolic extract of *N. indicum* latex for 24h exposure period

Parameters	Control		40% LC_{50} (5.80 mg/l)	80% LC_{50} (11.60 mg/l)
Total protein (µg protein/mg of tissue)	L	142.63±0.55(100)	125.51±0.14[a](88)	102.69±0.15[a](72)
	M	130.25±0.45(100)	117.23±0.22[a](90)	101.60±0.25[a](78)
Total free amino acid (µg/mg of tissue)	L	8.10±0.33(100)	9.72±0.18[a](120)	11.75±0.23[a](145)
	M	7.75±0.76(100)	8.68±0.16[a](112)	10.23±0.19[a](132)
DNA (µg/mg of tissue)	L	35.75±0.20(100)	30.39±0.18[a](85)	25.74±0.13[a](72)
	M	34.35±0.30(100)	30.92±0.21[a](90)	27.90±0.23[a](81)
RNA (µg/mg of tissue)	L	37.28±0.53(100)	29.82±0.37[a](80)	26.10±0.40[a](70)
	M	36.45±0.25(100)	31.35±0.18[a](86)	27.76±0.19[a](76)
Glycogen (mg glycogen/g of tissue)	L	3.10±0.34(100)	2.79±0.23[a](90)	2.64±0.33[a](85)
	M	2.25±0.43(100)	2.12±0.36[a](94)	1.96±0.29[a](87)
Pyruvate (µmoles pyruvate/g tissue)	L	3.05±0.23(100)	2.59±0.17[a](85)	2.20±0.23[a](72)
	M	2.85±0.41(100)	2.51±0.36[a](88)	2.14±0.39[a](75)
Lactate (mg lactic acid/g tissue)	L	2.57±0.12(100)	3.03±0.27[a](118)	4.37±0.28[a](170)
	M	2.10±0.15(100)	2.56±0.11[a](122)	2.98±0.23[a](142)
Protease (µmoles tyrosine/mg protein/h)	L	0.365±0.05(100)	0.435±0.02[a](119)	0.540±0.04[a](145)
	M	0.479±0.04(100)	0.537±0.01[a](112)	0.662±0.03[a](138)
Alanine aminotransferase (µmoles pyruvate/mg protein/h)	L	3.75±0.06(100)	5.25±0.09[a](140)	6.38±0.06[a](170)
	M	4.55±0.03(100)	5.92±0.06[a](130)	7.28±0.05[a](160)

Parameters	Control		40% LC_{50} (5.80 mg/l)	80% LC_{50} (11.60 mg/l)
Aspartate aminotransferase	L	1.90±0.03(100)	2.49±0.04[a](131)	3.10±0.09[a](163)
(µmoles pyruvate/mg protein/h)	M	2.10±0.04(100)	2.52±0.06[a](120)	3.15±0.07[a](150)
Acetylcholinesterase	L	2.20±0.15(100)	1.76±0.17[a](80)	1.54±0.20[a](70)
(µ mol 'SH'/min/mg protein)	M	1.85±0.13(100)	1.55±0.21[a](84)	1.22±0.16[a](66)
Lactic dehyrogenase	L	413.19±0.04(100)	315.21±0.06[a](85)	309.89±0.07[a](75)
(µ mol pyruvate/min./mg protein)	M	395.15±0.08(100)	355.63±0.05[a](90)	312.17±0.02[a](79)
Succinic dehydrogenase	L	49.62±0.23(100)	42.18±0.28[a](85)	37.22±0.35[a](75)
(µ mol reduced/min/mg protein)	M	57.21±0.20(100)	51.49±0.17[a](90)	47.48±0.29[a](83)
Cytochrome Oxidase	L	35.10±0.12(100)	30.54±0.18[a](87)	22.82±0.35[a](65)
(arbitrary unit/min/mg protein)	M	37.55±0.09(100)	34.55±0.12[a](92)	25.16±0.17[a](67)

*Values are mean ± SE of six replicates. Values in parentheses are % level with control taken as 100%. Data were analyzed through student's 't' test.
aSignificant ($P< 0.05$), when treated groups were compared with controls.

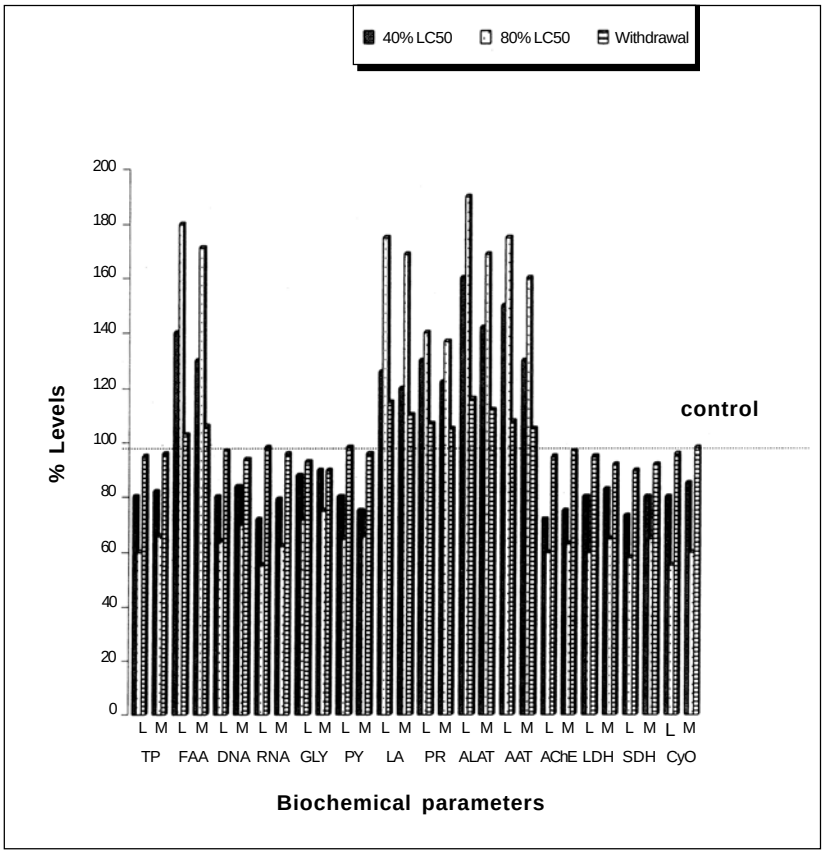

Fig. 1: Per cent level of biochemical parameters in the liver (L) and muscle (M) tissues of *Colisa fasciatus* fish after exposure to 40% (2.21 mg/l) and 80% (4.42 mg/l) of LC_{50} of ethanolic extract of *Nerium indicum* latex for 96 h exposure period and 7th day after withdrawal of treatment.
(TP=Total Protein, FAA=Total free amino acid, GLY=Glycogen, PY=Pyruvate, LA=Lactate, PR=Protease, ALAT=Alanine aminotransferase, AAT=Aspartate aminotransferase, AChE=Acetyl cholinesterase, LDH=Lactic dehyrogenase, SDH=Succinic dehydrogenase, CyO=Cytochrome oxidase)

Students 't' test and analysis of variance showed that these biochemical changes were significantly ($P<0.05$) time and dose-dependent.

Discussion

Changed behavioural responses can be taken as index of the stress felt by the fish exposed to NL_{EtOH}, by which they try to reduce excess entry of toxicant present in the water. Fishes of control group is free from such behavioural changes, which indicates NL_{EtOH} was responsible for above altered behaviour and fish mortality. Animal behaviour is a neurotropically regulated phenomenon, which is mediated by neurotransmitter substances[27]. Result also shows that the NL_{EtOH} caused significant inhibition in the activity of enzyme acetylcholinesterase of fish, this enzyme present in synaptic regions and mediates transmission of impulses by breaking acetylcholine into acetic acid and choline[28]. The acetylcholine at neural and neuromotar regions upon accumulation causes 'hyper-excitability[29], which in turn might also

influences behavioural pattern and cause twitching of muscle leading to paralysis of respiratory muscle and may lead to death of fish[29].

The steep slope values indicate that increased fish mortality occurred with relatively small increase in NL_{EtOH} dose. When the value of 't' ratio is greater than 1.96, it shows that regression is significant. Values of heterogeneity factor less than 1.0 denote that in the replicate tests of random samples the concentration response lines would fall within 95% confidence limits and thus the model fits the data adequately[30].

During stress condition, fishes needed more energy to detoxify the toxicants and to overcome stress. Carbohydrates are the primary and immediate source of energy[31]. In stress condition, carbohydrates reserve depleted to meet energy demand[28]. Depletion of glycogen may be due to direct utilization for energy generation, a demand caused by NL_{EtOH}-induced hypoxia[32].

Carbohydrate metabolism is mainly concerns to fulfill energy demand of animals by its aerobic and anaerobic segment[31]. The lactate level acts as an index of anaerobiosis, which was beneficial for animal to tolerate hypoxic condition[31]. Under stress condition, with the increases of lactate content there was a decrease in pyruvate content, which suggests a shift towards anaerobiosis as a consequence of hypoxia, leading to respiratory distress[28]. The prevalence of hypoxia during NL_{EtOH} treatment should have led to respiratory distress when it is forced to depend on deriving energy by anaerobiosis. Increase in lactic acid level in *C. fasciatus* suggests a possibility of flocculation of NL_{EtOH} (i.e. layering over gill membranes and filaments) thus altering the aerobic nature and a shift towards anaerobiosis. The increase in tissue lactate content may be due to its involvement in osmoregulation[28]. The decrease of pyruvate level may be due to its conversion to lactate or due to its mobilization to form amino acids, lipids, etc. synthesis in addition to its role as a detoxification factor[28]. Thus, it may be presumed that there is a tendency of shift in emphasis from the aerobic pathway to anaerobic pathway of fish respiration, to meet energy demands for the physiological and metabolic activities augmented by stress induced by NL_{EtOH}.

Since fish have a very little amount of carbohydrates so next alternative source of energy is protein to meet the increased energy demand[28]. The decrease in protein level in liver and muscle tissues may be due to meet out higher energy demands for metabolic purposes. Increase in level of free amino acids is due to breakdown of protein and also due to impaired incorporation of amino acids in protein synthesis and decline in nucleic acid level[33]. The decreases in total protein level and increases in total free amino acid level suggest the high protein hydrolytic activity due to elevation of protease enzyme activity.

Aspartate and alanine aminotransferase function as a link between carbohydrate and protein metabolism[34]. Under exposure of NL_{EtOH}, their activity were highly elevated in both the tissues of fish confirming the augmentation of stress. In present study, glycogen, which is ultimate energy source, decreases 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[31]. Since the amino acid level also increased so it is evident that both ALAT and AAT activities are being stepped up to be in line with the increasing energy demands. In liver and muscle tissue both, 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[31].

Lactic dehydrogenase (LDH) forms the center for a delicately balanced equilibrium between catabolism and anabolism of carbohydrates[28]. LDH mediates inter-conversion of lactate to pyruvate depending on the availability of NAD, Co-enzyme[28]. The decrease in lactate activity with a consequent increase in the levels of lactic acid suggests the predominance of anaerobic segment, glycolysis[28].

The general decrease in succinic dehydrogenase (SDH) activity during stress condition was associated with the inhibition of mitochondrial respiratory mechanism, or derangement in ultra structure, architectural integrity and permeability of mitochondria[35]. This prevents the transfer of electrons to molecular oxygen, resulting in the inhibition of SDH activity and shifting the aerobic metabolism to anaerobiosis[36].

Cytochrome oxidase transfers electrons to their final acceptor oxygen in electron transfer system (ETS) and thus produces ATP molecules[31]. Inhibition of CyO shows, NL_{EtOH} has a profound impact on the oxidative metabolism, possibly due to their influence on ETS. Decrease in CyO activity either the result of reduced availability of O_2, which in turns has

reduce the capacity of ETS to produce ATP molecules or should be due to their direct toxic impact[28].

Conclusion

Thus, it can be concluded that ethanolic extract of ***N. indicum*** latex has potent piscicidal activity and its ultimate mode of action is respiratory pathway of fish, which shift towards anaerobic segments and adversely affect their oxidative metabolism and inhibit energy production by suppressing ATP synthesis. Reversibility in their action was also another advantageous factor in their use.

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