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PISCICIDAL ACTIVITY OF ALCOHOLIC EXTRACT OF *NERIUM INDICUM* LEAF AND THEIR BIOCHEMICAL STRESS RESPONSE ON FISH METABOLISM

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Abstract

Laboratory evaluations were made to asses the piscicidal activity of ethyl alcohol extract of *Nerium indicum* leaf against predatory fish *Channa punctatus* and their ultimate mode of action on fish metabolism. Toxicity experiments show there was significant negative correlation between LC values and exposure periods i.e. LC_{50} value decreased from 66.32 mg/L (24h) to 44.96 mg/L (96h). Biochemical studies show that after exposure to sub lethal doses of alcoholic extract, pyruvate level and activities of acetylcholinesterase and cytochrome oxidase significantly decreased while lactate level and activities of alanine aminotransferase and aspartate amino transferase were significantly enhanced in both liver and muscle tissues of *Channa punctatus*. The alterations in all the above biochemical parameters were significantly ($P<0.05$) time and dose dependent. There was a significant recovery observed in all the above biochemical parameters, in both liver and muscle tissues of fish after the seventh day of the withdrawal of treatment Thus it was believed that alcoholic extract of *Nerium indicum* leaf can be used in aquatic environment for controlling predatory fish *Channa punctatus* population. Their piscicidal activity may be due to their adverse effect on respiratory as well as energy production of fish. The reversible nature of the piscicidal action could be advantageous in aquatic environment.

Keywords: Piscicidal activity, Alcoholic extract, *Nerium indicum,* fish metabolism, *Channa punctatus*.

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Introduction

The presence of predatory and weed fishes in culture pond is a serious problem for culturing edible fresh water fishes. These fishes adversely effect the cultured fish population in culture pond by sharing food and habitat of major cultivated carps. *Channa punctatus* is the common predatory fish which have low food value and due to there predatory nature, they engulf the fingerlings of cultured carp at several stages of their rearing (Jhingran, 1975) and thus adversely effect the cultured carp production and put a great loss to the fish farmer. For eliminating the unwanted population of *Channa punctatus* from cultured ponds, fish farmers made several efforts, in which the use of synthetic piscicides is most prominent (Marking, 1992). But due to their long term persistence in the water and fish body, they adversely affect both the quality of fish and their status (Cullen and Connell, 1992; Waliszewski et al., 1999). A better alternative of these harmful synthetic piscicides is environmentally safe plant origin piscicides which are less expensive, biodegradable, readily available, easy to handle and safe for mankind and environment both (Marston and Hostettmann, 1985; Singh et al., 1996). A large number of plants belonging to different families (Kulakkattolickal, 1989) and their products (Bhatia, 1970) have been used for controlling unwanted fish population not only in India but all over world (Tiwari, 2003). Chiayvareesajja et al. (1987) studied the toxicity of 221 plants in Thailand and found five plant species that are effective in killing predatory fishes. *Nerium indicum* (Family- Apocynace) is a common medicinal and rapidly growing garden plant of India. The plant is considered to be useful in the management and treatment of inflammation of gums, dysentery, bronchitis, asthma, and menorrhagia (Jain and Tarafder, 1970).

The toxicity of aqueous latex extract of *N. indicum* to the fresh water snails *Lymnaea acuminata* and *Indoplanorbis excustus* and on fishes *Channa punctatus* and *Colisa fasciatus* has been established (Singh et al., 1993; Singh and Singh, 2000), but the doses of aqueous extract were so high. So its purification is necessary for developing a new and effective herbal piscicide. But a new herbal piscicide cannot be directly used in fresh water bodies until their long term effect as well as their mode of action is well established. The present paper describes the piscicidal and biochemical activities of partially purified alcoholic leaf extract of *Nerium indicum* on common air-breathing predatory fish *Channa punctatus.*

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Materials and Methods

Collection and Preparation of extract of *Nerium indicum* leaf

Nerium indicum (Family- Apocynace) were collected locally from Botanical garden of D.D.U. Gorakhpur University, Gorakhpur and identified by Prof. S.K. Singh, Plant taxonomist, Department of Botany, D.D.U. Gorakhpur University, Gorakhpur, India.

Fresh leaves of *Nerium indicum* were dried in an incubator at 37°C, and with the help of mechanical device ground to powder. The dried powder (5.0 g) was extracted using soxhlet extractor with absolute ethyl alcohol (200 ml) for about 36 hours. The solvent was evaporated by vacuum pump to obtain the extract in dried form (1.45 g).. The dried extract was stored in airtight desiccators for further experiments.

Collection and storage of experimental animals

Fish, *Channa punctatus* (13.0±2.5 cm length, 12.5 ± 1.5 g body weight) were collected locally from the Ramgarh lake of Gorakhpur district and used as test animal. The collected fishes were maintained in glass aquaria containing 100L of de-chlorinated tap water for acclimatization to laboratory conditions at room temperature for one week. The aquarium water was aerated continuously and food was provided in the form of dried, powdered small prawn, goat liver etc. Water was changed at every 24 hour. The dead animals, (if any) were removed as soon as possible from test container to prevent water fouling. The physicochemical parameters of water were atmospheric temperature 29.0±1.0°C, water temperature 26.5±1.5°C, pH 7.1 to 7.3, dissolved oxygen 7.0 to 7.5 mg/L, free carbon dioxide 4.0 to 5.0 mg/L, bicarbonate alkalinity 103.0 to 105.0 mg/L (APHA/WEF, 1998).

Toxicity experiments

Toxicity experiment was performed by the method of Singh and Agarwal (1988). The fishes were exposed for 24h, 48h, 72h or 96 hour at four different concentrations of alcoholic leaf extract. Six aquaria were set up for each concentration and each aquarium contains ten fishes in 6L de-chlorinated tap water. Control animals were kept in similar condition without any treatment. Mortality was recorded at every 24h up to 96h exposure period. Fishes were considered dead if they failed to respond to stimulus provided 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 (POLO computer programme of Russel et al., 1977).

Treatment protocol for Dose-Response relationship

Channa punctatus were kept in glass aquaria containing 6L de-chlorinated tap water. Each aquarium contains ten experimental animals. Fishes were exposed for 24h or 96h exposure period to 40% and 80% of 24h or 96h LC_{50} doses of alcoholic extract of *N. indicum* leaf. Control animals were kept in similar condition without any treatment. After completion of treatment, fishes were removed from aquaria, washed with water and killed by severe blow on head. The liver and muscle tissue were quickly dissected out in ice tray and used for biochemical analysis. Each experiment was replicated at least six times. The values have been expressed as mean ±SE of six replicates. Student's't' test was applied to locate significant difference with controls (Sokal and Rohlf, 1973).

Pyruvate analysis

Pyruvate level was measured according to Friedemann and Haugen (1943). Homogenate (50 mg/ml) was prepared in 10% TCA. Sodium pyruvate was taken as standard. Result has been expressed as µg pyruvate/mg tissue.

Lactate analysis

Lactate was estimated according to the method of Huckabee (1961). Homogenate (50 mg/ml,) was prepared in 10% cold TCA and centrifuged at 1000 g for 5 minutes. Sodium lactate was taken as standard. Lactate content was expressed as µg/mg tissue.

Aspartate and Alanine Amino transferase activity

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activity were determined by the method of Reitman and Frankel (1957). Homogenate (50 mg/ml, w/v) was prepared in 0.25 M cold sucrose solution. Optical density was measured at 546 nm. The enzyme activity was expressed as µ moles of pyruvate formed/mg protein/hour.

Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was measured in the liver and muscle tissue by the method of Ellman et al. (1961), as modified by Singh and Agarwal (1982). Homogenate (50 mg/ml) was prepared in 0.1M-phosphate buffer, pH 8.0 for 5 min in an ice bath and centrifuged at 1000g for 30min at -4^0C. Protein estimation was done by the method of Lowry et al. (1951). Enzyme activity was expressed as µ mol 'SH' hydrolyzed/min/mg protein.

Cytochrome oxidase activity

This was measured according to the method of Cooperstein and Lazarow (1951). Homogenate (50 mg/ml) was prepared in 0.33M phosphate buffer, pH 7.4 for 5 minutes in ice bath and centrifuged at 10,000 g for 30 minutes at -4ºC and supernatant was used as enzyme source. Enzyme activity was expressed in orbitrary units/min/mg of proteins.

Withdrawal experiment

The fishes were exposed for 96h exposure period to 80% of the 96h LC_{50}.The one half of the animal was sacrificed and the activity of all above biochemical parameters were measured in liver and muscle tissues. The other half was transferred to fresh water, which was changed every 24h for seven days. Thereafter, all the above biochemical parameters were measured in liver and muscle tissue of fishes. Control animals were kept in similar condition without any treatment.

Results

Exposures to alcoholic leaf extract of *N. indicum* caused significant behavioural changes in the fish *C. punctatus*. On the introduction of leaf extracts, all the fishes immediately settled down at the bottom of aquarium. Within 5-10 minutes, the fishes felt suffocation and they came to the water surface for gasping the air. As exposure period increases surfacing phenomenon of fish's increases.

The rates of operculum movement, mucous secretion from skin and respiration through gill also increased. After some time the opercular movement of fish slowed down although they tried to stay at upper water surface and loss of body equilibrium was pronounced. Finally all their body activity decreased and they settled down at the base of the aquaria and died. Control animals were free from such behavioral changes.

Table 1, shows the effective dose ($LC_{10,\ 50\ and\ 90}$ values) of alcoholic extract *N. indicum* leaf at different exposure periods against *C. punctatus*. The results clearly indicated that mortality was both time and dose dependent. There was a significant negative correlation between LC_{50} values and exposure periods i.e. LC_{50} values decreased from 66.32 mg/l (24h) > 57.22 mg/l (48h) > 49.04 mg/l (72h) and > 44.96 mg/l (96h). Similar trends were also observed in case of LC_{10} and LC_{90} values (Table 1).

The slope values in toxicity (Table 1) were steep and heterogeneity factor less than 1.0 indicates that the result was found to be within the 95% confidence limits of LC_{50} 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.

Table 1: Toxicity (LC values) of absolute ethyl alcohol extract of leaf powder of *Nerium indicum* at different time intervals on the fish *Channa punctatus*.

Exposure periods	**Effective dose (mg/l)**	**Limits (mg/l)**		**Slope value**	**'g' factor**	**'t' ratio**	**Heterogeneity**
		LCL	**UCL**				
24h	LC_{10}=50.73	46.52	53.56				
	LC_{50}=66.32	63.46	70.44	3.00±1.61	0.08	6.84	0.15
	LC_{90}=86.71	79.40	100.96				
48h	LC_{10}= 42.87	39.16	45.61				
	LC_{50}= 57.22	54.89	59.81	3.22±1.18	0.05	8.66	0.44
	LC_{90}= 76.35	71.29	84.63				
72h	LC_{10}= 39.04	36.04	41.28				
	LC_{50}= 49.04	47.16	50.84	3.94±1.38	0.04	9.35	0.41
	LC_{90}= 61.61	58.74	65.79				
96h	LC_{10}= 36.50	33.42	38.70				
	LC_{50}= 44.96	43.12	46.63	3.15±1.68	0.05	8.40	0.29
	LC_{90}= 55.39	52.94	59.03				

- There was no mortality in control groups.
- Batches of ten fishes were exposed to four different concentrations of absolute ethyl alcohol extracts of *Nerium indicum* leaf.
- Concentrations given are the final concentrations (w/v) in aquarium water.
- Each set of experiment was replicated six times.
- Mortality was determined after every 24h up to 96 hour.
- Regression coefficient showed that there was significant ($P<0.05$) negative regression between exposure time and different LC values.
- LCL = Lower confidence limit; UCL = Upper confidence limit

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Exposure of fish to sub-lethal doses of 40% and 80% of 24 and 96 hour LC_{50} of alcoholic extract of *N. indicum* leaf revealed that pyruvate level, acetylcholinesterase and cytochrome oxidase enzyme activities were significantly ($P<0.05$) reduced while lactate level and ALT, AST enzyme activity were significantly ($P < 0.05$) enhanced in liver and muscle tissues of fish *C. punctatus* (Tables 2 and 3). Pyruvate level was reduced to 50% and 44%, acetylcholinesterase enzyme activity was inhibited up to 50% and 53%, cytochrome oxidase enzyme activity was inhibited up to 44% and 52%, lactate level were increased to 170% and 180%, ALT activity was increased to 185% and 173% and AST activity was increased to 150%and 160% of controls after treatment with 80% of 96 hour LC_{50} of alcoholic extract of *N. indicum* leaf in both liver and muscle tissue of fish, respectively (Table 3). After seven days withdrawal experiment of 80% of 96hour LC_{50} of alcoholic extract of *N. indicum* leaf (Table 3) showed significant ($P< 0.05$) recovery in the levels of pyruvate and lactate as well as the activitivities of enzymes ALT, AST, acetylcholinesterase and Cytochrome oxidase in both liver and muscle tissue of fish.

Discussion

Toxicity experiments showed that alcoholic extract of *N. indicum* leaf, caused significant behavioural changes in fish *C. punctatus*. The initial increase in opercular movement can be taken as index of the stress felt by the fish exposed to plant extract media. Subsequent decrease in opercular movement may be construed a passive response to prevent excess entry of extract molecule present in the medium to minimize damage to gill epithelium (Sambasiva, 1999). Fish of control group was free from such type of behavioural changes, so it was clear that only leaf moieties were responsible for the altered behaviour and fish mortality. Animal behaviour is a neurotropically regulated phenomenon, which is mediated by neurotransmitter substances (Sambasiva, 1999). From the results, it was evident that alcoholic leaf extract inhibit activity of enzyme AChE. This enzyme is present in synaptic regions and mediates transmission of impulses by breaking acetylcholine into acetic acid and choline (Sambasiva, 1999). The acetylcholine at neural and neuromotar regions upon accumulation causes 'hyper-excitability (Siva, 1980; Kabeer et al., 1980), which in turn might also influences behaviour pattern. Similar behavioural responses were also observed in organophosphate and carbamate pesticides exposed fishes (Gill et al., 1991). These compounds are cholinergic inhibitors and known as nerve

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poison (Stansely, 1993). Gill et al., (1991) also described that above behavioural anomalies are due to inhibition of enzyme acetylcholinesterase (AChE).

Table 2: Changes in Py, La levels and activities of enzyme ALT, AST, AChE and CyO in different tissues of *C. punctatus* after exposure to 40% and 80% of LC_{50} (24h) of alcoholic extract of *N. indicum* leaf for 24 hour.

	Tissue	**Control**	**40% LC_{50}** (26.53 mg/L)	**80% LC_{50}** (53.06 mg/L)
Py	Liver	2.40±0.03 (100)	2.16±0.03* (90)	1.92±0.02* (80)
	Muscle	1.83±0.01 (100)	1.57±0.02* (86)	1.37±0.04* (75)
La	Liver	1.63±0.05 (100)	1.79±0.01* (110)	2.12±0.03* (130)
	Muscle	1.34±0.02 (100)	1.54±0.04* (115)	1.78±0.05* (133)
ALT	Liver	3.93±0.06 (100)	4.52±0.07* (115)	5.11±0.06* (130)
	Muscle	3.11±0.05 (100)	3.42±0.03* (110)	3.79±0.02* (122)
AST	Liver	1.65±0.04 (100)	1.73±0.05* (105)	1.98±0.01* (120)
	Muscle	1.40±0.01 (100)	1.44±0.03* (103)	1.61±0.07* (115)
AChE	Liver	0.194±0.009 (100)	0.165±0.003* (85)	0.142±0.007* (73)
	Muscle	0.235±0.003 (100)	0.212±0.006* (90)	0.190±0.005* (81)
CyO	Liver	37.80±0.30 (100)	30.99±0.11* (82)	26.46±0.18* (70)
	Muscle	35.10±0.36 (100)	31.94±0.20* (91)	28.08±0.14* (80)

- Values are mean ± SE of six replicates.
- Values in parentheses are % change with control taken as 100%.
- Data were analysed through student's test.
- *, Significant ($P < 0.05$), when treated groups were compared with controls.
- Py and La were expressed in µg/mg while ALT/AST, AChE and CyO expressed in µ moles pyruvate/mg protein/h, µ mol 'SH'/min/mg protein and orbitrary units/min/mg of protein respectively.
- Py: Pyruvate, La: Lactate, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, AChE: Acetylcholinesterase, CyO: Cytochrome oxidase

Mortality caused by the leaf extracts showed a clear significant positive correlation between dose and mortality. The positive correlation between dose and mortality in present study was noted because increase concentration of extract in aquarium water resulted in more intake or entry of active moieties

in the fish body. This trend is also dependent on several factors such as, rate of penetration, nature of slope, variability of active moieties, etc. (Goodmann et al., 1985).

The steep slope values indicates that there is a large increase in the mortality of fishes with relatively small increase in the dose of different treatments given in Table 1.When the value of the 't' ratio is greater than 1.96 the 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. The index of significance of potency estimation 'g' indicates that the value of the mean is within the limits at all probability levels (90, 95, and 96) as it is less than 0.5 (Rand and Petrocelli, 1988).

Table 3: Changes in pyruvate, lactate levels and activities of enzyme ALT, AST, AChE and CyO in different tissues of *C. punctatus* after exposure to 40% and 80% of LC_{50} (96h) of alcoholic extract of *Nerium indicum* leaf for 96 hour and 7th days after withdrawal.

	Tissue	**Control**	**40% LC_{50}** (17.98 mg/L)	**80% LC_{50}** (35.97 mg/L)	**7th days after withdrawal**
Py	Liver	2.23±0.02 (100)	1.65±0.01*(70)	1.18±0.03*(50)	2.23±0.07 (95)
	Muscle	1.74±0.03 (100)	1.04±0.05*(60)	0.77±0.02*(44)	1.57±0.09† (90)
La	Liver	1.55±0.04 (100)	2.17±0.03*(140)	2.64±0.08*(170)	1.63±0.03† (105)
	Muscle	1.22±0.06 (100)	1.77±0.02*(145)	2.20±0.05*(180)	1.31±0.01† (107)
ALT	Liver	3.90±0.03 (100)	6.24±0.01*(160)	7.22±0.03*(185)	4.29±0.06† (110)
	Muscle	3.10±0.01 (100)	4.65±0.05*(150)	5.36±0.01*(173)	3.32±0.07† (107)
AST	Liver	1.60±0.04 (100)	2.08±0.03*(130)	2.40±0.01*(150)	1.68±0.02† (105)
	Muscle	1.33±0.02 (100)	1.82±0.06*(137)	0.67±0.03*(160)	1.37±0.09† (103)
AChE	Liver	0.190±0.005 (100)	0.133±0.007*(70)	0.095±0.003*(50)	0.177±0.001†(93)
	Muscle	0.232±0.007 (100)	0.139±0.001*(60)	0.123±0.005*(53)	0.211±0.004† (91)
CyO	Liver	36.77±0.34(100)	22.06±0.21*(60)	11.78±0.29*(44)	33.83±0.33† (92)
	Muscle	33.40±0.45(100)	23.38±0.26*(70)	17.37±0.17*(52)	30.06±0.26† (90)

- Details are same as shown in table 2.
- Significant (P< 0.05), when withdrawal groups were compared with treated groups.

• Py and La were expressed in µg/mg while ALT/AST, AChE and CyO expressed in µ moles pyruvate/mg protein/h, µ mol 'SH'/min/mg protein and orbitrary units/min/mg of protein respectively.

Py: Pyruvate, La: Lactate, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, AChE: Acetylcholinesterase, CyO: Cytochrome oxidase

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Carbohydrate metabolism is broadly divided into anaerobic segment or glycolysis in which break down of glucose or glycogen through Embden Meyerhaf pathway occur and aerobic segment that consist oxidation of pyruvate to acetyl co-A to be utilized through citric acid cycle (Lehninger, 1978). The end product of glycolysis under anaerobic condition in tissue is lactic acid where as 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 indicator of anaerobiosis, which might be beneficial for animal to tolerate hypoxic condition (Thoye, 1971) under toxicant exposure condition. Pesticides are also inhibiting energy production by suppressing aerobic oxidation of carbohydrate leading to energy crisis in animals (Kohli et al., 1975). In the case of liver and muscle both aerobic and anaerobic condition are likely to operate depending on availability of molecular oxygen and other physiological needs imposed by other factor.

Under stress condition, with the increases in lactate content there was a decrease in pyruvate content in all the tissue. 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 (Domsche et al., 1971; Siva, 1980). The increase in tissue lactate content may be due to its involvement in osmoregulation. During stress condition there was a decrease in osmotic regulation of internal body media of animal by loss of both mono and divalent cations which are compensated for in the animals with the increase of organic ions like lactate etc. (Kabeer et al., 1984). 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 (Sathya, 1983). Both the aspartate and alanine aminotransferase 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 (Knox and Greengard, 1965; Watts and Watts, 1974). In present study, we find that both aminotranferases activity were higher in liver tissue than muscle tissue of control fishes (Tables 2 and 3). This suggests that liver tissue is very efficient in utilizing amino acid for metabolic purposes.

Under exposure of alcoholic extract of *N. indicum* leaf, the activity of both aminotransferases was highly elevated in both the liver and muscle tissue, which indicated the augmentation of stress as a

consequences of diethyl ether extract of *N. indicum* leaf. Stress in general is known to elevate aminotransferase activities (Natarajan, 1985). This also conforms with the nature of function of aminotransferases, where they respond to any stress or altered physiological condition (Knox and Greengard, 1965). Such a situation takes place in the present study, fishes need more energy to tolerate stress condition 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. Here both ALT and AST activities are being stepped up to be in line with the increasing energy demands to full fill their demand through amino acids. In liver and muscle tissue both, ALT predominates over AST where the feeding of amino acids into energy cycle is more through alanine - pyruvate pathway representing anaerobic tendency of the tissues.

Cytochrome oxidase is a haemoprotein, and act as a terminal component of the respiratory chain in mitochondria. It transfers electrons to final acceptor, oxygen. Thus being a terminal link in electron transfer system (ETS) it produces ATP molecules there by influencing other cellular metabolic process. Cytochrome oxidase activity was observed to decrease in the liver and muscle tissues of *Channa punctatus* after exposure to sub-lethal doses of alcoholic extract. Inhibition in cytochrome oxidase activity supports that alcoholic extract shows a profound impact on the oxidative metabolism, possibly due to their influence on respiratory process such as the electron transport system (Sambasiva, 1999). Decrease in cytochrome oxidase activity might be either the result of reduced availability of oxygen, which in turns has reduced the capacity of the ETS to produce ATP molecules or should be due to the direct impact of the toxicant. Stevans et al. (1972) reported that anticholinesterase compounds are known to usually inhibit the function of the cytochrome oxidase in the ETS. So, the toxicant affects Kreb's cycle there by diminishing the rate of the ETS and oxidative phosphorylation, resulting in less ATP synthesis. The decrease in tissue respiratory rates, the activity of oxidative enzyme and accumulation of lactic acid suggest the tendency of shift in the metabolism of carbohydrates more towards anaerobic dependence than aerobic oxidation.

Thus alcoholic leaf extract of *N. indicum* may be considered as a potential new piscicide for control of predatory fish *Channa punctatus* from carp culture ponds during pre-fertilization. The reversible nature of the toxic action of the extract on fish would be an added advantage in its use.

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