Biosafety of *Azadirachta indica* (A. Juss) leaves extracts on certain biochemical parameters of *Labeo rohita*

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ABSTRACT

The side-effect of *Azadirachta indica* (A. Juss) leaf extract on certain biochemical parameters of a freshwater fish, *Labeo rohita* was studied for a period of 25 days at sublethal concentration. The median lethal concentration (1.035 g/L) of *A. indica* for 24 h was calculated and 1/10th of the toxicant (0.1035 g/L) was taken for sub-lethal study. During the exposure period the glycogen content in the liver and muscle of leaf extract treated fish increased up to the 15th day and then declined, whereas the protein level decreased in the liver and muscles throughout the study period. The alterations of these biochemical parameters can be effectively used as non-specific biomarkers against plant extract toxicity stress and also help safer usage of plant extracts in aquaculture farms.

Key words: *A. indica*, sub-lethal toxicity, glycogen, protein, *L. rohita*

INTRODUCTION

The presence of predatory and weed fishes in culture pond is a serious problem for culturing edible freshwater fishes in India. This has adversely affected the development of fish production (Tiwari and Singh, 2003) and to overcome this problem the use of synthetic piscicides is most common practice in many aquaculture farms (Gribgratok, 1981; Marking, 1992). These synthetic piscicides due to their long-term persistence in the water and fish body adversely affect the quality of fish and their status (Cullen and Connell, 1992; Waliszewski *et al.*, 1999) and also results contamination of aquatic environment. To solve these problems, studies are being carried out on the feasibility of using biopesticides or plant extracts. In recent years, the use of medicinal plants as effective alternatives of synthetic pesticides and fertilizers has gained importance especially to combat problem both in fish and aquatic environment because they are highly toxic to the target pests.

Neem, *Azadirachta indica* (A. Juss), one of the most versatile multipurpose plant species well known for it's insecticidal, biomedical and pharmacological properties (Govindachari, 1992; ICAR, 1993; Biswas *et al*., 2002), and hence, traditionally used to treat many diseases (Van Der Nat *et al.*, 1991). Recent studies show that neem leaf powder can be used to deliver pesticides (Singh *et al.*, 2010). The extracts of *A. indica* has been used successfully in aquaculture systems to control fish predators (Dunkel and Ricilards, 1998), parasites (Winkaler *et al*., 2007), antimutagenic (Farah *et al.*, 2006) and pathogenic bacteria such as *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Escherichia coli* and *Myxobacteria* spp (Das *et al.*, 2002).

Although neem extract is considered of low toxicity towards non-target organisms, water extracts of the various parts of neem plant caused many problems like respiratory problems, delayed growth of fishes and also interfere with the maintenance of their homeostasis and thus affect their performance (Singh and Singh, 1980a, b; Gopal *et al.*, 1981; Omoregie and Okpanachi, 1992, 1997). Such results indicate that neem extracts added to water may cause disturbances on fish. Consequently it is important to determine the effects caused by these products using certain parameters of fish. Biomarkers for water pollution are early diagnostic tools for biological effect measurement and environmental quality assessment (Cajaraville *et al.*, 2000). Among the variety of biomarkers adopted in ecotoxicological investigations, there is notable interest in parameters related to biochemical alterations. Plasma glucose, liver and muscle glycogen and protein responses appear particularly suitable for measuring stressful levels of pollutants and have long been used as indicators of stress in fish (Hattingh, 1976; Srivastava and Srivastava, 1988; Ramesh, 2001).

The effects of neem on non-target organisms have been studied in terrestrial ecosystems, however little attention has been focused on the effects of neem in aquatic environments. Hence, in the present investigation the toxic

effect of neem extract on certain biochemical parameters of a cultivable fish (*L. rohita*) was studied to fill up this lacuna.

MATERIALS AND METHODS

Experimental animal and water

Fingerlings of *L. rohita* in the weight range of 8.0 ± 0.5 g and body length of 8.0 ± 1 cm were obtained from Tamil Nadu Fisheries Development Corporation Limited, Aliyar Fish Farm, Aliyar, Tamil Nadu, India. They were safely brought to the laboratory and acclimatized for 20 days in a large cement tank (1000 litre capacity) prior to the experiment. During the acclimatization period, fish were fed *ad libitum* with rice bran and groundnut oil cake in the form of dough once daily. Water was renewed (one third of the water) daily and feeding was withheld 24 h before the commencement of the experiment. In the present study tap water free from chlorine was used and the water had the following physico-chemical characteristics (APHA, 1998); temperature (26.3 ± 2°C), pH (7.2), dissolved oxygen (6.2 mg l^{-1}), total hardness (90 mg l^{-1}, as $CaCO_3$), salinity (0.4 ± 0.02 ppt). Before the start of the experiment, fish were randomly divided into two groups which were housed in 200^{-l} aquaria with tap water which was continuously aerated.

Preparation of aqueous neem leaf extracts

The leaves of *A. indica* (A. Juss) were collected in and around Bharathiar University campus, dried and finely chopped. To prepare the aqueous extract the leaves were dissolved in water at a concentration of 25 g of dried leaves per liter of water for 24 hours at room temperature (Cruz *et al.*, 2004). The mixture was filtered and the extract (25 g/L) was used immediately in the experiments, in different dilutions.

Determination of 24 h LC_{50} value of neem leaf extract

Static acute toxicity (24 h) test was conducted to determine the LC_{50} value of neem leaf extract toxicity considering the limitations of laboratory facilities. Different concentrations of the neem leaf extract i.e., 0.25, 0.50, 0.75, 1.0, 1.25, 1.50 ppm were prepared from the stock and used to find out the LC_{50} value for 24 h. For each concentration 10 fish randomly selected from the stock were introduced and kept in separate glass tanks (120cm×80cm×40cm). To each concentration a control (normal tap water without leaf extract) with three replicates was maintained. The mortality/survival of fish was recorded after 24 h. The dead fish were removed from the tank immediately. Feeding was withheld during the bioassay experiment. The concentration at which 50 percent mortality of fish occurred after 24 h was taken as the medium lethal concentration (LC_{50}) for 24 h, which was 1.035 ppm. The LC_{50} concentration for 24 h was calculated by the probit analysis method of Finney (1978). One-tenth value of the LC_{50} concentration of neem leaf extract for 24 h (0.1035 ppm) was taken as the sublethal concentration (Sprague, 1971).

Sublethal toxicity studies

For sublethal toxicity tests 200 fingerlings were selected and divided into two groups with 100 fish in each aquarium. Each group was exposed to sublethal concentration of the neem leaf extract (0.1035 ppm). A similar set up was also maintained as control. During sublethal studies, fish were fed *ad libitum* before water replacement. The water in the aquarium was renewed for every 24 h and the aqueous leaf extract of neem concentration (0.1035 ppm) was added daily in the treatment group in order to maintain constant concentration. Experiment was conducted for 25 days and no mortality was observed during the above treatment period. At the end of 5, 10, 15, 20 and 25th days of exposure, fish were randomly selected from experiment and control aquarium for the analysis. Liver and muscle samples were collected from each group for the glycogen and protein assays.

Estimation of glycogen, protein in liver and muscle

Estimation of liver and muscle glycogen was estimated by anthrone method (Samseifter *et al.*, 1949) and liver and muscle protein estimation was done according to the method of Lowry *et al.* (1951).

Statistical analysis

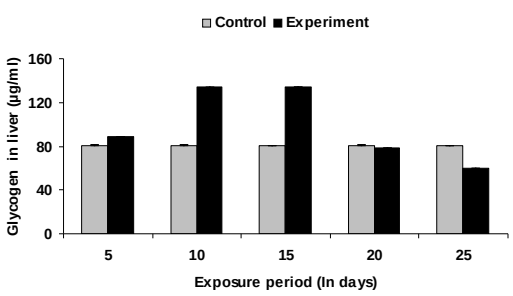
The data were analysed statistically at $P < 0.05$. To test their significance the *t*-values were calculated by Student's *t*-test.

RESULTS

The glycogen content in the liver increased up to the 15th day showing a maximum change of 66.18 percent (Fig 1). After 15th day the level of glycogen decreased showing a decrease of 3.31, 21.51 percent at the end of 20th and 25th day, respectively. The glycogen content of muscle of fish increased up to the 15th day showing increase of 16.20, 37.92 and 53.81 percent at the end of 5th, 10th and 15th day, respectively (Fig 2). After the 15th day, glycogen content in muscle decreased showing 10.29, 31.79 percent at the end of 20th and 25th day, respectively (Fig 2). The liver protein level decreased throughout the study period showing a minimum percent change of 7.16 and a maximum percent change of 46.63 at the end of 5th and 25th day, respectively (Fig 3). The protein level in muscle decreased

throughout the study period showing a percent decrease of 9.12, 17.37, 25.14, 36.92 and 41.34 at the end of the 5th, 10th, 15th, 20th and 25th day, respectively (Fig 4).

(1)



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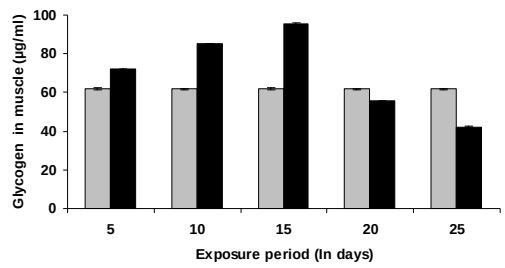

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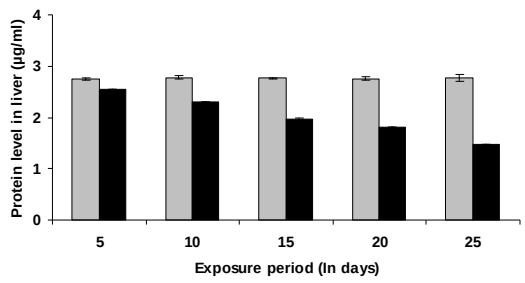

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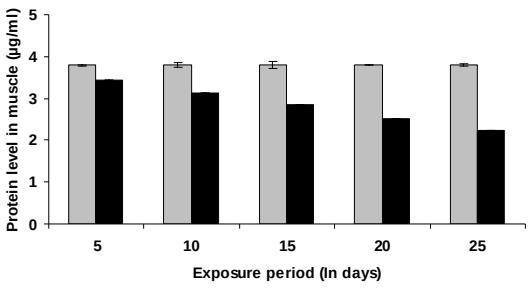

Fig 1-4. Glycogen and protein levels in liver and muscle of control and aqueous leaf extracts of *A. indica* treated fish (0.1035 ppm for 25 days). Values are means ± SE of five individual observations; Values are significant at 5% level.

DISCUSSION

Comparisons of the sensitivity of different fish species to neem are questionable, since the amount of active compounds in a given weight of neem varies widely with the part of the plant (Luo *et al.*, 1999), its place of origin or even the individual tree (Isman *et al.*, 1990; NRC, 1992). Compared to other synthetic insecticides used in fish farming, such as carbamates and organophosphates, neem based products are certainly less toxic to fish (Winkaler *et al.*, 2007). Neem was also shown to be less toxic to *P. lineatus* than the herbicide Trifluralin (24 h LC_{50} = 0.25 mg l^{-1}) and the insecticide Azodrin (96 h LC_{50} = 28.28 mg l^{-1}) as reported by Martinez and Cólus (2002). In the present study the LC_{50} 24 h value was 1.035 g/L which is more or less similar to the findings of the previous works. In the present investigation, during acute treatment significant behavioural changes like increase in opercular movement, mucous secretion, erratic movement etc., were noticed in neem leaf extract exposed fish. The fish of control group were free from any such type of behavioural changes indicate that only leaf moieties were responsible for the altered behavioral changes. Tiwari and Singh (2003) also noticed similar behavioural changes in *Channa punctatus* exposed to *Nerium indicum* leaf extract.

Carbohydrates are the primary and immediate source of energy (Tiwari and Singh, 2006); under stress, carbohydrate reserves get depleted to meet energy demand. Depletion of glycogen may be due to direct utilization for energy generation, a demand caused by aqueous extract-induced hypoxia (Kohli *et al.*, 1975).

Glycogen the ultimate energy source, decreases, resulting in higher demand for carbohydrate and their precursors to keep the glycolytic and Krebs's cycles at sustained levels to cope with energy demands during stress condition. Reduction in glycogen level is thought to be the result of greater stress the organs experienced during the process of detoxification of active moieties and their metabolites. Liver glycogen levels are depleted during acute hypoxia or physical disturbances in the fish (Heath and Fritechard, 1965). In the present study the decrease of glycogen in liver and muscle may be due to direct utilization of energy generation, a demand caused by aqueous extract induced hypoxia. The significant increase in liver and muscle glycogen after 15th day in the present investigation indicate suppression of insulin secretion or the action of neem leaf extract on the endocrine system. Since fish have a very little amount of carbohydrates (Sambasiva Rao, 1999), the next alternative source of

energy is protein to meet the increased energy demand. The depletion of protein fraction in liver and muscle might have been due to their degradation and possible utilization for metabolic purposes. During chronic period of stress, proteins act as a source of energy. Bradbury *et al*. (1987) pointed out that the decreased protein content might also be attributed to the destruction or necrosis of cells and consequent impairment in protein synthesis machinery. Tiwari and Singh (2006) reported that the decreases in protein level in the liver and muscle of fish exposed to neem extract might have resulted from high protein hydrolytic activity due to elevation of protease enzyme activity in both the tissues. In the present study the depletion of protein level in liver and muscle tissues during sublethal treatment might have resulted from their degradation and possible utilization of degraded products for metabolic purposes.

CONCLUSION

The present study indicates that leaf extract of *A. indica* has caused significant alterations in glycogen and protein content of liver and muscle of fish *L. rohita*, which might be of help to establish the safer usage of aqueous extracts of *A. indica* in aquaculture farms.

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