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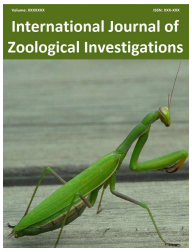

Botanical Pesticide *Nerium indicum* Alters Prolactin Cells of Stinging Catfish, *Heteropneustes fossilis*

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Abstract: Effect of *Nerium indicum* leaf extract exposure on histo-cytology of prolactin cells were investigated in *Heteropneustes fossilis.* Fish were subjected to 11.27 mg/L (80% of 96 h LC_{50}) and 2.81 mg/L (20% of 96 h LC_{50}) of *Nerium indicum* leaf extract for short- and long-term, respectively. After sacrificing the fish, blood was collected on 24, 48, 72 and 96 h in short-term and after 7, 14, 21, and 28 days in long-term experiment and analyzed for serum calcium levels. Pituitary glands were fixed on these intervals. No alteration was noticed in serum calcium levels of *H. fossilis* at 24 h. Serum calcium levels decreased from 48 h to 96 h. In prolactin cells of fish no histological alteration was observed throughout the short- term exposure. *Nerium indicum* caused decrease in serum calcium levels of *H. fossilis* on day 14 which progresses till 28 days. Up to 14 day following treatment with *Nerium indicum* leaf extract no histological change in the prolactin cells of *H. fossilis* has been noticed. These cells exhibit slight degranulation on day 21, however, the nuclear volume remains unchanged. Further degranulation of these cells has been noticed following 28 day exposure. Nuclear volume records an increase and at some places, vacuolization and cytolysis are also observed.

Key words: Botanical pesticide, *Nerium Indicum*, prolactin cells, Calcium, *Heteropneustes fossilis*, Teleost

Introduction

The use of persistent organochlorines like DDT and of the acute toxic organophosphates has led to hazardous effects on environment and human beings. Therefore, attention was directed towards the development of alternative chemicals. Botanical pesticides based on plant extracts such as nicotine, rotenone and pyrethrum have been commonly employed for pest control during the earlier half of this century. Recently, these have been used as alternatives to synthetic pesticides for agricultural pest management. It is believed that botanical pesticides will reduce the undesirable side effects of synthetic pesticides and help to preserve the natural environment.

Many plant species from different families which possess number of compounds such as - saponins, tannins, alkaloids di- and tri-terpenoids have been reported to cause pesticidal activity and used for control of 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; Singh et al., 1996, 1998, a, b; Dubey et al., 2010). However, some botanicals may be dangerous for humans and they can be very toxic to natural enemies. Before applying a new botanical pesticide in large scale, its effect on ecosystem should be tested.

Nerium indicum (family– Apocynaceae) is amongst the most poisonous plant of India. *Nerium indicum* leaves produced clinical effects in humans which are mainly cardiac and gastrointestinal, including nausea, vomiting, salivation and diarrhoea (Hardin and Arena, 1974). *Nerium* is also used as rodenticide and insecticide. Attempts had been made by few investigators to evaluate the toxicological aspects of various parts of *Nerium* species on few organisms– fish (Tiwari and Singh 2003, 2004, 2005, Singh and Singh, 2002; Prasad et al., 2009, 2011, 2013a, b), mammal (Oryan et al., 1996; Longford and Boor 1996; Ada et al., 2001; Haeba et al., 2002; Aslani et al., 2004; Saravanan et al., 2004; Soto-Blanco et al., 2006; Barbosa et al., 2007), insect (el-Shazly et al., 1996) and molluscs (Singh et al., 1993, Panigrahi and Raut, 1994; Wang et al., 2006).

Among vertebrates prolactin has a physiological role in certain biological features such as stimulation of the pegion crop, stimulation of mammary gland and post-ovulatory corpus luteum in some mammals and induction of water-drive and land-water integumentary changes in the urodeles (Meites and Nicoll 1966; Vellano et al., 1967). However, fish prolactin does not elicit these responses. A role of prolactin during organogenesis in zebrafish (Nguven et al., 2008) and in nuptial colouration in female fish has been reported (Skold et al., 2008). Removal of pituitary gland caused a decline in the serum/plasma calcium level of freshwater fish (Wendelaar Bonga and Pang, 1989; Srivastav et al., 1998). In this study, we have attempted to investigate the effects of *Nerium indicum* leaf extract on the histological alterations in the prolactin cells of a teleost, *H. fossilis.* To the best of our knowledge, the effects of botanical pesticides on prolactin cells of fish have not been reported yet.

Materials and Methods

Adult freshwater stinging catfish *Heteropneustes fossilis* (both sexes, body weight 32 – 44 g) were collected locally. Healthy fish showing no external signs of injury and disease were selected for experiments and were acclimatized to laboratory conditions (under natural photoperiod 11.46 – 12.18 and temperature 26.74 ± 2.11 °C; pH 7.26 ± 0.09; hardness 167.97 ± 5.69 mg/L as $CaCO_3$; dissolved oxygen 7.85 ± 0.36 mg/L) for 15 days in dechlorinated tap water. The study was approved by the Animal Research Ethical Committee of DDU Gorakhpur University, Gorakhpur.

In the present study, *Nerium indicum* leaf extract was used. The 96 h LC_{50} value of *Nerium indicum* leaf extract (14.0 mg/L for the fish *H. fossilis*) have been reported by Prasad et al. (2009). In short-term exposure the fish were subjected to 11.27 mg/L of *Nerium indicum* leaf extract (80% of 96 h LC_{50} value). In long term exposure the fish were subjected to 2.81 mg/L (20% of 96 h LC_{50} value) of *Nerium indicum* leaf extract. Simultaneously, a control group was also run for comparison by using the tap water containing ethanol. Fish were kept in groups of 10 in 40 L media. *Nerium indicum* leaf extract was weight and stock solution (50 mg/ml) was prepared in 100% ethanol. Six fish were sacrificed (anaesthesized with MS 222) on each time intervals from control and experimental (*Nerium indicum* exposed) groups after 24,

48, 72 and 96 h in short-term exposure and after 7, 14, 21 and 28 days in long-term experiment.

Blood samples were collected by sectioning of the caudal peduncle of fish. The sera were separated by centrifugation at 3500 r. p. m. and analyzed for calcium levels (calcium kit, RFCL Limited India). After the collection of blood samples, the pituitary glands along with the brain were fixed in aqueous Bouin's fluid and Bouin's-Hollande fixatives for histological studies. Tissues, thus fixed were routinely processed in graded series of alcohols, cleared in xylene, and then embedded in paraffin wax. Serial sections were cut at 6 µm and stained with Herlant tetrachrome for light microscopic examination (Olympus CH 20i). Photo-micrographs were taken with the aid of Olympus E 420 camera

Nuclear indices (maximal length and maximal width) of prolactin cells were determined (50 nuclei were measured per specimen; thus 300 nuclei were measured from six specimens) with the aid of ocular micrometer. When there is degeneration of prolactin cells only the nuclear indices of intact cells were measured. The nuclear volume was calculated as volume = $4/3\ \pi\ ab^2$, where 'a' is the major semiaxis and 'b' is the minor semiaxis.

All data are presented as the mean ± S.E. of six specimens and student's 't' test was used for the determination of statistical significance. In all studies, the experimental group was compared to its specific time control group. Two-way Analysis of Variance (ANOVA) was used for multiple group comparisons.

Results

In short term experiment the serum calcium levels of *H. fossilis* exhibits a decline after 48 h following exposure to *Nerium indicum* leaf extract. This decrease continues till the end of the experiment (96 h) (Fig 1). Analysis of variance indicated that the level of serum calcium were significantly different between groups (between intervals $F = 9.38$, $P<0.0001$; between treatment $F = 55.78$, $P<0.0001$).

The structural details of prolactin cells of control fish (Fig. 2) are almost similar to that of normal fish.

Nerium indicum leaf extract caused no change in the histological structure of prolactin cells of *H. fossilis* throughout the short-term experiment. Analysis of variance indicated that in short-term experiment the nuclear volume of prolactin cells were not significant (between intervals $F=7.78$, $P<0.0001$, ns; between treatments $F= 0.53$, $P<0.471$, ns)

In long-term experiment, *Nerium indicum* leaf extract provoked a decline in the serum calcium levels of *H. fossilis* on day 14. The levels progressively decline till the close of the experiment (28 days) (Fig 3). Analysis of variance indicated that the levels of serum calcium for long-term were significantly different between groups (between intervals $F = 16.46$, $P<0.0001$; between treatment $F = 113.7$, $P<0.0001$).

Up to 14 day following treatment with *Nerium indicum* leaf extract no histological change in the prolactin cells of *H. fossilis* has been noticed. These cells exhibit slight degranulation on day 21 (Fig. 4), however, the nuclear volume remains unchanged. Further degranulation of these cells has been noticed following 28 day exposure with *Nerium indicum* leaf extract (Fig. 5). Nuclear volume records an increase (Fig. 6) and at some places, vacuolization and cytolysis are also observed (Fig. 7). Analysis of variance indicated that in long-term experiment the nuclear volume of prolctin cells were significantly different between groups (between intervals $F = 9.82$, $P <0.0001$; between treatment $F = 1.72$, $P< 0.198$).

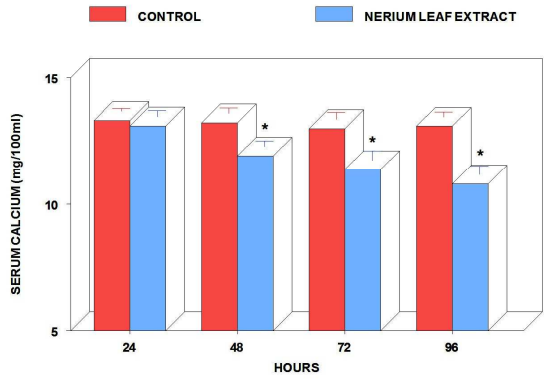

Fig. 1: Serum calcium levels of short-term *Nerium indicum* leaf extract treated *H. fossilis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences (P<0.05) from control.

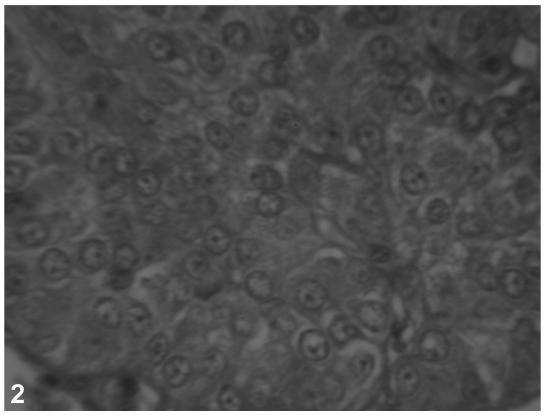

Fig. 2: Prolactin cells of control *Heteropneustes fossilis*. Herlant tetrachrome X 800.

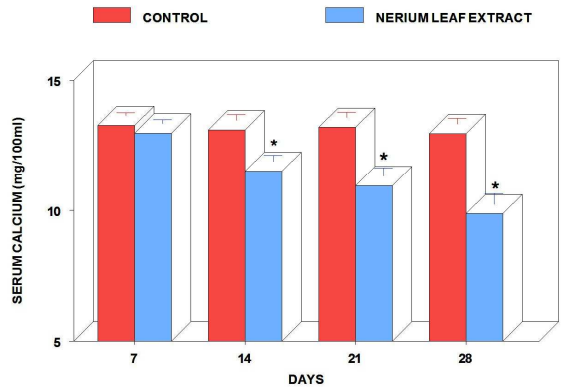

Fig. 3: Serum calcium levels of long-term *Nerium indicum* leaf extract treated *H. fossilis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences (P<0.05) from control.

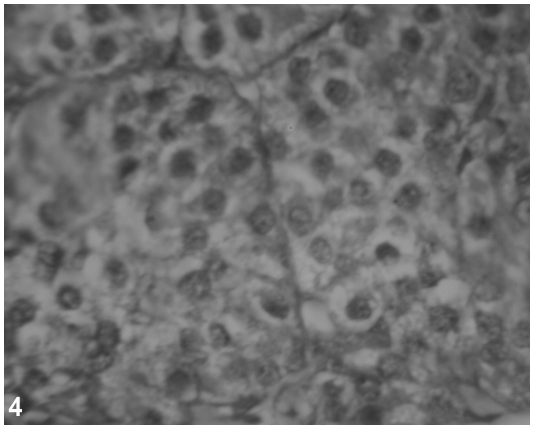

Fig. 4: Prolactin cells of 21 days *Nerium indicum* leaf extract treated *Heteropneustes fossilis* showing slight degranulation. Herlant tetrachrome X 800.

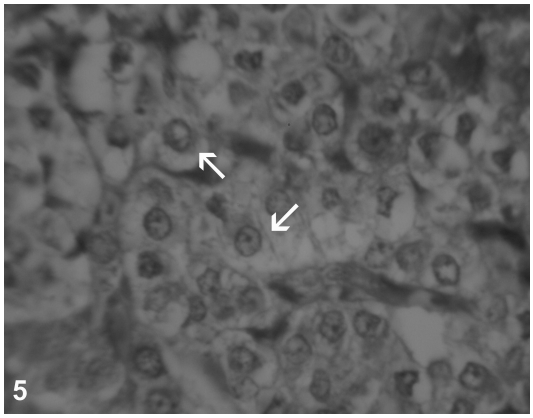

Fig. 5: Prolactin cells of 28 days *Nerium indicum* leaf extract treated *H. fossilis* showing further degranulation (arrow). Herlant tetrachrome X 800.

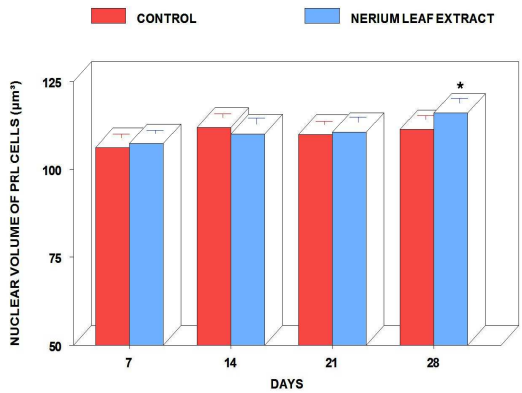

Fig. 6: Nuclear volume of prolactin cells of long-term *Nerium indicum* leaf extract treated *Heteropneustes fossilis.* Each value represents mean ± S.E. of six specimens. Asterisk indicates significant differences (P<0.05) from control.

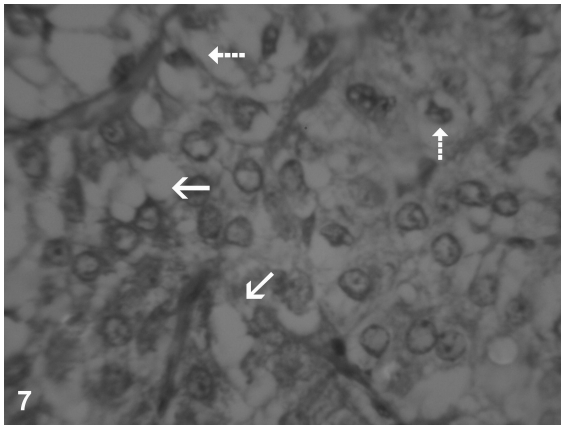

Fig. 7: Prolactin cells of 28 days *Nerium indicum* leaf extract treated *Heteropneustes fossilis* showing vacuolization (arrow) and cytolysis (broken arrow). Herlant tetrachrome X 800.

Discussion

Exposure of the freshwater catfish *H. fossilis* to *Nerium indicum* leaf extract resulted in degranulation and increased nuclear volume of prolactin cells. There are only few studies regarding the effects of toxicants on the activity of prolactin cells (James and Wigham, 1986; Fu, 1989; Mishra et al., 2008, 2009; Srivastav et al., 2010). The present study is in agreement with the reports of earlier investigators as they have also recorded hyperactivity of prolactin cells of fish in response to exposure of toxicants- cadmium (tilapia- Fu, 1989), metacid (catfish- Mishra et al., 2008), cypermethrin (catfish- Mishra et al., 2009) and deltamethrin (catfish- Srivastav et al., 2010). Contrary to this, cadmium injection to rainbow trout failed to elicit any effect on prolactin cell activity (James and Wigham, 1986). The observations of Meredith et al. (1999), Thangavel et al. (2005, 2010) and Ramesh et al. (2009) strengthens the present study as these investigators have noticed increased levels of prolactin in toxicant exposed fish. Bone demineralization has been reported in cadmium exposed carp (Koyama and Itazawa, 1977; Muramoto, 1981) and lead exposed fish *Catla catla* (Palaviappan et al., 2010).

Prolactin elicits hypercalcemia in various species of fishes (Pang et al., 1978; Wendelaar Bonga and Flik, 1982; Flik et al., 1986, 1994; Wendelaar Bonga and Pang, 1991) by altering gill epithelium permeability (Dharmamba and Meatz, 1972; Clark and Bern, 1980; Wendelaar Bonga et al., 1983). Hyperactivity of prolactin cells in *Nerium indicum* leaf extract treated fish could be attributed for maintaining the ionic balance in blood of treated *H. fossilis* through action of prolactin on gills, kidney and bones.

Conclusion

It can be concluded that *Nerium indicum* can severely affect the physiology of calcium homeostasis in fishes as we have noticed alterations in serum calcium content as well as cytological changes in prolactin cells of the freshwater fish, *H. fossilis*. Calcium is important for several physiological processes including reproduction. Prolactin elicits hypercalcemia in various species of fishes by altering gill epithelium permeability hence any alteration in calcium and prolactin cells causes physiological disturbances which might affect seriously the normal vital functions, growth rate and their survival in nature. Hence, the botanical pesticides should be used with caution near fish inhabiting water reservoirs.

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