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Assessment Of Acute Toxicity And Behavioral Responses In Cat Fish *Clarias Batrachus* (Linnaeus) Exposed To Biopesticide Neem Oil

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Abstract –

The extensive use of pesticides in aqua farms and disagreement to scientific management practices have negative impact on production of aquaculture, dangerous outbreaks of diseases, development of pesticides resistance in harmful pests, and accumulation of pesticide residues in freshwater fishes and environmental pollution. As a conclusion, a need has been felt by the aquaculturists and agriculturists as well as human and aquatic animal health management skillful to find a suitable another treatment in place of harmful pesticides and chemicals. Biopesticide has come to be identified as a handy and viable alternative to pesticides, as it is effective, economical, non-resistance forming, eco-friendly and farmer-friendly. Neem oil is a biopesticide and most effective for harmful insects and pests which can be extracted from most parts of the neem tree, but the seeds hold highest concentration of the insecticidal compound. In the present study, acute toxicity of neem oil was investigated and 96 h LC_{50} value calculated as 0.848 ml /L to the freshwater cat fish, *Clarias batrachus*. The experimental fishes showed alterations in their responses when concentration of neem oil and time of exposure increased day by day. This study may help to determine the neem oil toxicity to the fish in aquatic ecosystem and to understand the mode of its action to fish behavior.

Key words – Biopesticide- Neem oil, Behavioral changes, LC_{50} value, Freshwater fish *Clarias batrachus*

1 - Introduction -

Pesticides usage has increased tremendously day by day. In spite of the fact that pesticides uses helps to improve crop yields and productivity, it is crucial to note when pesticides are used enormously; they come with negative impacts–so that they can cause environmental pollution. Pesticides can run off through agricultural fields or percolate by the ground to enter ground water resources. Pesticides spraying on crops, or using pesticides in the soil for protection from the pest and insects, can leave some residue on agricultural crops and plants products. These harmful pesticides absorbed by the root of plants and crops and then reach in body of human and other animals when they eat them. Ultimately the pesticides after being used reaches to aquatic ecosystem and have been found to be very much toxic to non-target living beings, especially aquatic animals (Nwani *et al.*, 2010). These poisonous substances may accumulate in food chain and produce serious ecological and health problems (Ali Sadeghi and Imanpoor, 2015). Rapid

urbanization, agricultural development and industrialization in India have resulted in high impact on both quantity and quality of water (CPCB, 2010). The use of traditional insecticides like persistent organophosphates and organochlorines is dangerous to environments and biota. But most natural chemicals available have limited effectiveness except neem oil. Everyone wants to protect our food, our families and our environment from the poisonous pesticide toxicity. So it has led to increasing interest in the use of alternative agricultural chemicals (Devine and Furlong, 2007; Kumar *et al*., 2011) such as biopesticides or plant derived pesticides. Plant-derived pesticides have potential as environmental friendly pesticides (Boeke *et al*., 2004).

Recently biopesticides are increasingly accepted by agriculturists owing to the current trend for organic things that uses fewer agrochemicals. Neem oil is a biopesticide and made pressed from the fruits and seeds of the neem (*Azadirachta indica*). Neem oil is the most valuable of the economically available products of neem and is used for medicine and organic farming. It consists of mainly steroids, triglycerides and various triterpenoids. Azadirachtin is the most well known and studied triterpenoid in neem oil and is the most active of these growth transformers (limonoids), finding at 0.2–0.4 % in the seeds of the neem tree (Metcalf, 2007). The triterpenoid is nimbin which has been valued with some of neem oil's qualities as an antifungal, antipyretic, antiseptic, and antihistamine (Kraus, 1995). Neem oil formulation also widely used as a biopesticide for agriculturists (Emken Tyler, 2021) and for organic farming because it repels a wide range of pests including the thrips, locust, nematodes, whiteflies, mites, mealy bug, beet armyworm, aphids, the cabbage worm, mushroom flies, leafminers, fungus gnats, beetles, moth larvae, caterpillars and the Japanese beetle (Isman, 2006; Mishra *et al*., 1995).

Neem oil can be used as a household pesticide for mosquitoes, ant, bedbug, cockroach, housefly, sand fly, termite and snail as repellent and larvicide (Puri, 1999). Neem oil is not known to be harmful for beneficial insects such as butterflies, honeybees and ladybugs and also birds, earthworms, mammals if it is not concentrated directly their food source or into their area of habitat. Neem oil acts as an antifeedant and hindered the action of the insect molting hormone ecdysone. Biopesticides containing neem oil are biodegradable, so they apparently do not increase the toxic residues level in the environment, unlike other synthetic pesticides (Menezes *et al*., 2004).

Fisheries have always played an essential role in providing food to people in India as well as over the world and freshwater aquaculture give more than 90% of total aquaculture production. India stands seventh position in the world in the production of fish, yet supplies only 8 Kg of fish per person in case of 11-12 Kg prescribed by WHO Standards. The air breathing cat fishes as these grow in marshes and swamps can fill up the shortage. India has wide resources of marshes and swamps, which can be utilized for air-breathing fish cultivation (Patnaik and Patra, 2006). Like other biopesticide neem oil may be less toxic for fish comparison to other pesticides and various assays are needed for its toxicity evaluation. For short term lethality assays various fish species have been used based on their ecological relevance, ease of culture and economic importance. The freshwater cat fish *Clarias batrachus* belonging to the class Actinopterygii was chosen for the present study due to availability throughout the season, wide distribution in the freshwater environment and easy acclimation to laboratory conditions.

This work has been carried out to determine the acute toxicity of biopesticide neem oil on cat fish *Clarias batrachus* (Linnaeus) and to observe behavioural responses after neem oil exposure. Animal behavior is observed an early toxicity indicator as it may allow integrated measures of

neurotoxicity in the existence of contaminants (Scott and Sloman, 2004). The aim of this study was to determine the toxicity of the neem oil, a product extensively used in fish-farms as alternative of poisonous synthetic pesticide for the freshwater fish *Clarias batrachus*.

2 - Material and methods –

I - Experimental fish and Biopesticide -

Different studies have been conducted in assessing the toxicity of pesticide and biopesticide to the aquatic biota especially fishes. The cat fish *Clarias batrachus* (normal weight 60 - 120 g and 20 - 25 cm length) were obtained from a local fish supplier. Experimental fishes were brought to the departmental laboratory of zoology and were kept in glass aquaria measuring 75cm x 37cm x 37cm. These fishes were carefully observed and before stocking treated with 2% $KMnO_4$ solution for one minute to get rid of any dermal infection. Prior to experimentation test fishes acclimatized for two weeks. The test fishes were fed with commercially available fish food twice a day at a rate of 2.5% body weight and draw off uneaten feed and fecal matter was done daily. Different chemicals as well as biopesticides belonging from various groups are available in the market but out of them neem oil was selected for present work because less records were found in toxicity effect on fish. Neem oil is a neem based biopesticide used in this study which is manufactured by Coromandel Agro Products and Oils Pvt. Ltd.

II - Acute toxicity bioassay and determination of sub lethal concentration

The experimental fishes were separated in to four groups (A, B, C, and D) in four aquaria to assess the lethal concentration of neem oil and one control group of fish maintained simultaneously. Each aquarium contained 20 L of water and consists of ten fishes in each group. Test fishes were starved for 24 h before starting the experiment. The lethal concentration of neem oil noted as the LC_{50} at which 50% mortality of test fishes take place. To find the mortality of *Clarias batrachus* fishes, test was done using four concentrations (10, 15, 20 and 25 ml) of neem oil and 10 fishes were exposed for each concentration and 20 L of water in each aquarium. These four concentrations were taken on basis of previous research works. The data were collected and used to calculate median lethal concentrations (LC_{50}) value. Concurrently behavior responses of the fish were also marked and recorded during the exposure period.

3 - Results

The aim of this work was to investigate the lethal concentration value of neem oil biopesticide and behavioral responses after 24, 48, 72 and 96 hrs for freshwater fish C*larias batrachus*. The acute toxicity analysis is short term exposure to experimental animals under the laboratory condition. The mortality of the experimental animal is the most observable response to find out LC_{50} concentration of the specimen. Mortality was noted after 24, 48, 72 and 96 h of exposure and dead fishes were taken out immediately from aquaria. Number of dead fishes was increased with increases of exposure of time and concentration of neem oil which is showing in Table – (1). In case of control group the swimming patterns and the behavioral alterations of the fish were normal and no mortality found in this group. To determine the actual LC_{50} values by probit analysis method, the concentrations obtained from the test were changed into log concentrations and their empirical value of probit were assessed by using Finney's table (Finney, 1971). A regression line graph was plotted between probit values and the log concentrations for various

exposure times. The results of the probit analysis by log dose concentration (X) and probid values (Y) are presented in Fig - (1). The 96 h LC_{50} value of neem oil for *Clarias batrachus* was obtained as 0.848 ml /L (Table -2). It is also observed that the toxic effects of Neem oil increases as concentrations increase and cause the acute toxicity in fish as compare to control.

During acute treatment, the cat fish *Clarias batrachus* exhibited behavioral responses such as erratic swimming, loss of reflex, profuse secretion of mucus, hypersensitivity, air gulping etc. Behavioral responses are the most sensitive indication of potential poisonous effects. Impact of various pesticides on the behavior of fishes have been studied by different workers (Anita et al., 2010; Bhat *et al.*, 2012; Marigoudar et al., 2009; Nagaraju *et al.*, 2011). Fishes showed a number of behavioral alterations when they were exposed to various concentrations. The movement of fish's operculum initially increases and then regularly decreases. Reduced opercula movement generally helps in decreasing absorption of pesticide through gills. Loss of balance and abnormal swimming was caused by the deficiency in muscular and nervous coordination which may be due to accumulation of acetylcholine in synaptic and neuromuscular junctions (Rao *et al.*, 2005). A thick layer of mucus was observed all over body of the test fish, representing the fish slimier. The experimental fishes were swimming with the belly upside and in zigzag movement. Erratic and parallel movements were also noted in the fish, shows loss of equilibrium while in control, the fish was swimming usually without loss of equilibrium. The fish becomes highly excited at higher concentration and was noted to hit the walls of aquarium at a very fast speed. The experimental fish C*larias batrachus* is less sensitive to neem oil at low concentration and highly reactive at high concentration.

Table 1– Survival number and percentage mortality of *Clarias batrachus* after 96 hours of treatment with different concentrations of biopesticide neem oil

Group	Concentration (ml/20L)	No of fishes	No, of fishes died	No. of fishes survived	Percentage Mortality
A	10	10	1	9	10%
B	15	10	3	7	30%
C	20	10	6	4	60%
D	25	10	8	2	80%

Table 2– Toxicity evaluation of neem oil to *Clarias batrachus* specifying fiducial limits and LC_{50}

Experimental animal	Test compound	Regression equation	Variance	Fiducial limits	Mean of F. Limits (M)	LC_{50}
Clarias batrachus	Neem oil	Y=1.168+4.67(X-5.35)	0.002	1.23292(+) 1.22508(-)	1.229	16.96ml/20L (or) 0.848ml/L

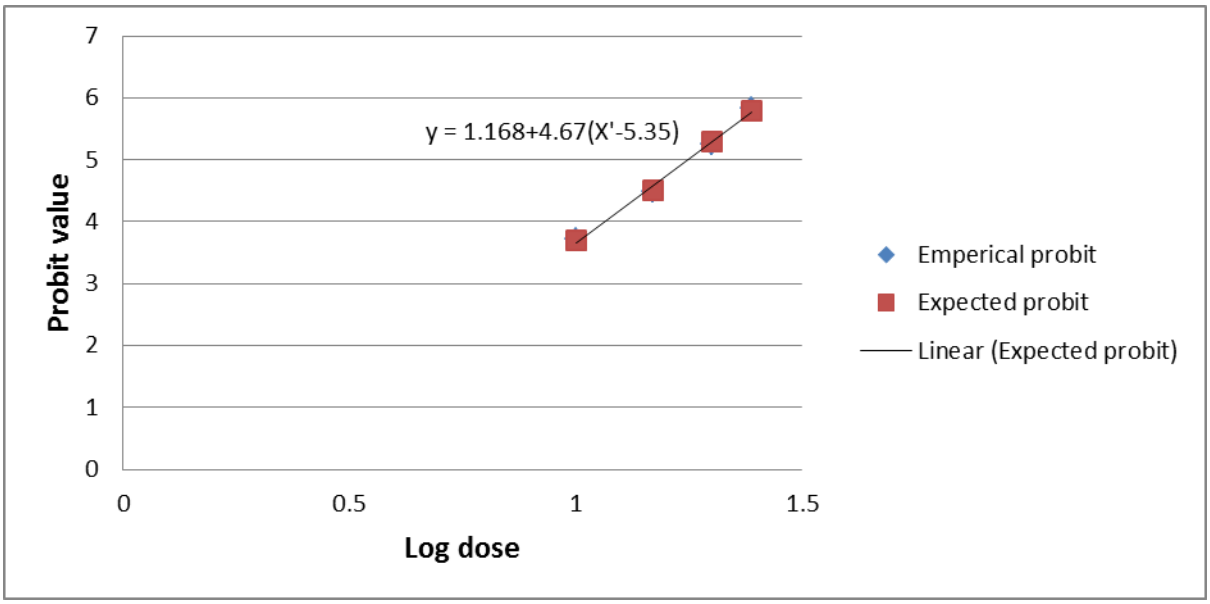

Fig 1– Determination of LC_{50} Log-dose Probit regression analysis after treatment with different doses of neem oil to *Clarias batrachus*.

4 - Discussion

The acute toxicity values of different biopesticides for various fish species have been observed earlier by many observers. In the present investigation the 96 hours LC_{50} value noted of neem oil was 0.848 ml /L which is approximately near to the value reported by Jothigayathri *et al.*, (2019) as 0.9 ml/L in fish *O. mossambicus* and much higher than the value obtained by Ahalavat and Hasan (2018) as 24.2 ppm to fish *Labio rohita* after exposure of neem-on. They also recorded that Floraguard pesticide is highly toxic to neem-on to freshwater fish *Labeo rohita* and noted abnormal behavioral patterns during the whole experiment. Cagauan *et al.*, (2004) observed lethal concentration of neem to Nile tilapia *Oreochromis niloticus L.* and mosquito fish *Gambusia affinis* Baird and Girard as 12.4 ml/L and 8.31 ml/L respectively and the equivalent 96 h LC_{50} values were 2.57 and 3.0 ml/L). Gholami *et al.*, (2015) assessed the chronic effects of a bio-pesticide (Neem Azal) on biochemical parameters of grass carp (*Ctenopharyngodon idella*) and 96 h LC_{50} value was found to be 0.73 mg/L of the active ingredient (azadirachtin) of the biopesticide. The differences in obtained values of 96 hours LC_{50} for different species were may be due to size, age and health or the used physiological parameters of water (Eaton and Gilbert, 2008). Davoodi (2012) noted that a dose of 0.17 ml/L of Neem Gold (EC 0.03%) resulted in the maximum death of fish and heavy mortality within 72 h in common carps. It can be consider that the level of active compounds in a given weight of neem differ widely with the part of the plant.

In the present investigation the exposed fish showed loss of equilibrium, erratic swimming and hyperactivity with the increment of time of exposure and concentration (Saravanan, 2011). The process of mortality begins with changes in behavior such as from normal movements to erratic

movements and finally the fish die similarly observed by Syafriadiman (2009). Dhara and Karmakar (2016) investigated acute toxicity of neem (*Azadirachta indica A. Juss*) leaf extracts on the fish, *Channa gachua* (Ham) and ethological responses were also observed. The 24, 48, 72 and 96 hours LC_{50} values were 21.80, 19.59, 13.95 and 11.18 g/l respectively for *C. gachua* recorded by them. The behavioral changes in fish treated with neem oil provide new vistas to observe the nature of toxicity as well as physiological level of the fish under exposure. Das *et al.,* (2002) have observed the acute toxicity of neem in the Indian major carp's fingerlings i.e., *Cirrhinus mrigala*, *Labeo rohita* and *Catla catla* and the 96h LC_{50} values were noted to be 2.78ppm, 2.36ppm, 2.04ppm respectively. Ishi and Patil (2017) evaluated toxic effect of biopesticide New Tech to freshwater cyprinid *Danio aequipinnatus* and LC_{50} values for 24, 48, 72 and 96 hours was found to be 2.1698 ppm, 1.9183 ppm, 1.7649 ppm and 1.5549 ppm respectively. Winkaler *et al.,* (2007) studied on freshwater fish Prochilodus lineatus and noted acute lethal and sublethal effects of neem leaf extract. They estimated the 24 h LC_{50} as 4.8 g/L. of neem leaf extract for juveniles *P. lineatus.* Their results indicate that although neem extract is less toxic to *P. lineatus* than other synthetic insecticides used in fish-farming it does cause functional and morphological less changes in this fish species. Similarly, Israel *et al.,* (2008) evaluated LC_{50} value at 96 hours of azadirachtin which is a *A. indica* based pesticide for Poecilia reticulate and found that LC_{50} value of azadirachtin has higher than that of the deltamethrin, a chemical pesticide. Septiningsih *et al*., (2021) evaluated median Lethal (LC_{50} 48hours) with Azadirectha biopestisides in low salinity in post larva tiger shrimp (*Penaeus monodon*) and noted there were 100% deaths at a biopesticide concentration of 89,995 ppm. Biopesticides are not much harmful to the environment (Ghimerya *et al.,* 2009) comparative to synthetic pesticide.

Bhat *et al.,* (2012) evaluated 96 h LC_{50} value of neem on and dichlorvos was found to be 42.66 ppm, 16.71 ppm respectively and concluded that plant based pesticide is less disastrous and more environment friendly. Similarly Asifa Alam *et al.,* (2014) studied on different concentrations of acetamiprid (10, 11, 12, 13, 14 and 15mg/L) and *Azadirachta indica* leaves extract (250, 500, 750, 1000 and 1050 mg/L) to determine their LC_{50} values on fish *Labio rohita*. They noted LC_{50} values of *A. indica* leaves extract and acetamiprid were 785.4 mg/L and 11.62 mg/L, respectively. Ajitha and Jayaprakas (2017) assessed impact of Menma (cassava based biopesticide) along with a Nimbecidine (neem based biopesticide) and Imidacloprid (neonicotinoid insecticide) on the fresh water fish, *O. niloticus*. They were calculated LC_{50} values of all three insecticides and their 1/5th values (Menma - 0.15 ppm, Nimbecidine - 0.014 ppm , Imidacloprid - 0.012 ppm) were taken as the sub lethal doses and noted slightly changes due to the presence of Nimbecidine biopesticide, no changes due to the menma biopesticide in biochemical and hematological parameters of fish *O. Niloticus*. Ramachandramohan and Mamatha (2015) studied impect of neem oil on skin of freshwater fish *G. Giuris* and revealed that the acute toxicity of neem oil affects mildly and cause minute effects on the structure of fish skin compared to other organophosphorus pesticides. Govindachari and Gopalkrishnan (1998) noted insect antifeedant and growth controlling activities of neem seed oil. Therefore, the use of

neem oil in fish industry should be promoted as it increases the ability to fight decreases and stop the growth of pathogenic organisms.

A study was carried out by Sumana and Rathore (2020) to investigating the use of Neem Oil for the control of aquatic insects in nursery ponds while Pereyra *et al.,* (2012) observed toxicity of neem oil, a potential biopesticide against invasive mussel Limnoperna fortune. Neem oil plays a strong antimutagenic role in fish which could further provide to study its beneficial impacts in humans. Similarly Braga *et al.,* (2021) studied literature review of latest in vivo toxicity of *A. indica* and divided it in two major sections—mammalian toxicity and aquatic animal's toxicity in which each related to neem's application as a potential new therapeutic drug or as a pesticide respectively. Maranho *et al.,* (2014) tested the acute toxicity of neem biopesticide (*Azadirachta indica A. Juss*) with zebra fish *Danio rerio* and chronic toxicity of neem biopesticide (*Azadirachta indica A. Juss*) with *Daphnia magna*. Similarly Ansari and Ahmad, (2010) evaluated toxicity of neem based pesticide neem gold and synthetic pyrethroid lambda-cyhalothrin on zebra fish *Danio rerio* (Cyprinidae) and suggested that biopesticides are less poisonous and more safer to our environment as compared to toxic synthetic pesticides. Hassain *et al.,* (2007) noted LC_{50} value of a neem biopesticide (triology) after the 96 hrs on grass carp *Ctenopharyngodon idella* was found to be 112 ppm. Imtiyaz *et al.,* (2012) evaluated the 96 hrs LC_{50} value on fish *Labeo rohita* due to the exposure of Matrine (Kethrin), was to be 21.68 ppm. Gavit and Patil (2016) tested the LC_{50} value of Acephate on the *Puntius sophore* after 96h, was to be 1117 ppm. Ansari and Sharma (2009) considered Achook, a neem based pesticide to be poisonous to zebra fish. Teresa *et al*., (2021) concluded that neem extracts have no or very less acute toxicity on mammalians. Hence these safety qualities and their well-known pharmacological properties, neem tree and its compounds present high commercial values and can be observed serious candidates to new natural drugs therapies. Biopesticides are target specific, biodegradable and eco-friendly as potential alternative to synthetic pesticides in addressing different pest management issues (Bibliography, 2007).

5 - Conclusion

In the present study it can be concluded that biopesticide is less toxic than other pesticides to the freshwater fish *Clarias batrachus* and other freshwater fishes because LC_{50} value with the neem oil is very high compare to other pesticide toxicity. Acute toxicity study is first step to determining the water quality essentials of fish and the study reveal the toxicant concentrations which cause fish mortality even at short term exposure (Pandey, 2005). If lower doses are used sub acute and sub chronic toxicity on these aquatic animals can be eliminated. Thus, due to these safe properties and their well-known pharmacological properties, neem tree and its neem oil present a high commercial importance and can be declaimed serious candidates to new natural medicine therapies. It can be also revealed that although biopesticide (neem based) is considered as less poisonous and eco-friendly but precautions must be taken when these biopesticide used in fish habitation because it can affect organisms life. From the observations presented and highlighted in this assessment it is clear that neem oil presented the lowest toxicity for aquatic

animals while other synthetic pesticides or chemicals presented moderate to high toxicity. Toxicity estimation is important to find out toxicant limit as well as safe concentration so that in future there will be less impair to aquatic fauna.

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