CHAPTER-1

Isolation and characterization of neurohormones and toxicity evaluation
Introduction
Introduction:-

In all multicellular animals, the nervous and endocrine system is so coordinated that the activities of the various organs and tissues in the body functions as individuals. The nervous system serves for rapid communication. For this complicated chains, interconnected neurons are necessary for transmission of transient impulses, together with the highly localized production of chemicals such as adrenalins and acetyl cholines, while the endocrine system uses circulatory body fluids to carry its chemical messengers to specific target organs. The nervous and endocrine systems are strictly interdependent. All developmental and physiological responses results from changes in the concentration of circulating hormones caused by nerve impulses originating by the stimulation of particular sensory cells.

In molluscan neurosecretory hormones are of the most important in controlling the growth and development both through their trophic effects upon epithelial endocrine gland and also by a direct intervention in metabolic processes. But neurosecretory hormones are also concerned in the regulation of much more temporary physiological events, such as heart
beat, color change and movements of retinal pigments, hydro mineral regulation, sexual differentiation and reproduction.

About few decades ago hormonal research on molluscan was in its infancy and it was assumed that the factors controlling the physiology and biochemistry of these molluscan are few and relatively simple. But active application in this field by many researchers forced the hormonal mechanisms in controlling the metabolism in molluscan are as complex as they are in vertebrates. Neurosecretory cells are morphologically similar to neurons with axons, dendrites nissals, granules and neurofibrils. They are also able to transmit nervous impulses, but their axans do not innervate effector organs. The materials they synthesize are released from the ends of the axans and exert a biological effect some distance away. The neurosecretory cells are neurons which also produce hormones.

In molluscan various physiological processes like lipid metabolism, carbohydrates metabolism, respiration, osmoregulation, mineralization and metamorphosis are under hormonal control as neurosecretory cells with other specialized cells are much modified in form, structure and behavior to suite them to their function. Histologically there are three groups of neurosecretory cells such as mediodorsal cells, laterodorsal cells and
caudodorsal cells. From cytomorphologically each group is having two types of neurosecretory cells ‘A’ and ‘B’ types. ‘A’ types of neurosecretory cells are larger in size with large sized nucleus. ‘A’ type of neurosecretory cells are less in number in each group while ‘B’ types of neurosecretory cells are smaller in size and number of ‘B’ cells are more in each group. The cell body is rounded to oval in shape with centrally located nucleus. Pollutants even they are in minute concentration can impair the functioning of the neuroendocrine system. However it is well known that in molluscan, various physiological processes are under hormonal control. Neurosecretory cells are production site of several hormones.

Most of the pesticides known to affect the neural functions at various level (Anderson et. al. 1970). Majority of insecticide interfere with the function of nervous system is primarily due to inhibition of the enzyme acetyl cholinesterase resulting in a disturbance of cholinergic. Organophosphate and Carbamate pesticide bind to the action site of acetyl cholinesterase and prevent breakdown of acetylcholine (Fukuto 1971). Gundevia and Ramamurthi (1972) observed various histopathological changes in the neurosecretory cells and retrocerebral endocrine complexes
of *Hydrophilus olivaceous* after treatment with Dimecron and Dieldrin. Number of workers like Coppage and Mathews (1994) observed the inhibition of acetylcholinesterase activity in pink shrimp *Penaeus durarum* after exposure to organophosphate pesticide. Nagabhushanum et.al (1972) described the cytological evidence that toxic substance increase in the quantity of neurosecretory materials in the medulla terminalis X –organ of the fiddler crab *Barytelphusa cunicularis* which indicate that the Arochlor inhibits release but not synthesis of neurohormones.

For decades mussels have been used as a sentinel species to monitor pollution in the aquatic environment (Foster and Bates 1978), Blackmore and Wang (2003). Study of toxic substances present in water and their adverse effects including mortality in aquatic organism, increase with the growing awareness of the hazards of discriminate water pollution. The toxicological studies of pollutants are gaining more significance in recent time and worldwide attempts have been made to identify a hazard from toxic chemical present or released in aquatic environment. The toxicity study is essential to find out toxicants limit and safe concentration, so that there will be minimum harm to aquatic fauna in the near future. Among the several aspects of toxicity studies the bioassay constitutes one of the most
commonly used methods in aquatic environmental studies with suitable organisms. The necessity of determining the toxicity of substances to commercially aquatic forms at the lower level of the food chain has been useful and accepted for water quality management. Several studies have been conducted in assessing the toxicity of pesticides to the aquatic biota. Attempt has been made to use certain snails species as bioindictors of metal contamination of fresh water bodies. The present investigation has been planned and executed to assess the impact of folicure pesticide on physiological aspects of freshwater snail *Lymnea auricularia*. The toxicity of pesticide and its rate of uptake from solution depend upon their oxidation states. So it is important to analyze their concentration which has become an important tool in assessing the environmental contamination and ecotoxicology.

The major objective of aquatic toxicological studies in laboratory were to identify the mechanism of toxicity and to predict safe contaminant concentration in the environment Jacobson (1993) reported that acute toxicity bioassay is the first stage of aquatic toxicology. The toxicology of folicure pesticide on the fresh water snails *Lymnea auricularia* is expressed in term of Lc50 values. This value represents the amount of
poison per unit weight which kills 50% of the particular population of the animals species employed for the tests (Finney 1971). The relationship between the dose of a compound and it is expressed as parts per million (ppm).

Hazardous substances released in large quantities from industrial, agricultural and urban activities and their by discharge into the aquatic ecosystem where it cause deleterious effect on aquatic organisms Kulkarni (1993) reported the acute toxicity of cadmium to the fresh water bivalve *Lamellidens marginalis*. Piansiri and Pachance (2008) observed the toxicity bioassay of the fresh water snail *M. martensi* exposed to mercury and cadmium. Since last three decades acute toxicity bioassay in general are useful in measuring the toxicity of different pollutants to aquatic organisms.

Since many workers directed the studies towards the toxicity evaluation ( Rao 1981, Bhavani and Dawood 2003 and Gomot ‘A’1997).The reaction and survival of aquatic organism, under toxic conditions depend upon several factors such as temperature, salinity, dissolved oxygen, pH, seasons and time of exposure to the toxicants. Ecotoxicological research on selected pollutants requires interdisciplinary...
efforts considering physicochemical, molecular, toxicological, physiological and ecological processes whereas practical aspects of ecotoxicology are focused on regulatory issues as registration of chemicals in standardized tests, understanding of toxicological phenomenon in a variety of biota, population and ecosystem.

Review of literature reveals paucity of information on acute toxicity of folicure pesticide on fresh water snail *Lymnea auricularia*. Hence the present probe has been selected to evaluate the acute and subacute toxicity effects on fresh water snail *Lymnea auricularia* as bioindicator, of local importance from Maharashtra state in Girna River.
Material and Methods
Material and Methods:

The fresh water snail *Lymnea auricularia* were collected from Chankapur Dam near Kalwan Dist. Nashik and brought to the laboratory. Mud and algal material attached to the snails were removed and acclimated for 3-5 days to laboratory conditions in plastic troughs. After acclimatization medium sized active snails were selected for experiments.

Environmental parameters such as temperature 24°C, pH 6.8, Dissolved Oxygen 4.8 mg/L and Total hardness 175-180 mg/L was maintained throughout the experimental period.

The snails were divided in to several batches and experiments were carried out in two steps.

a) **Isolation of neurohormones** and

b) **Toxicity evaluation.**

a) **Isolation and characterization of Neurohormones**

Extracted-30 cerebral ganglions were dissected out and non-nervous tissues were carefully removed. The ganglions were homogenized thoroughly in 2 ml ammonium acetate buffer (pH 8.5, 50mM). Homogenate was centrifuged for 30 min.at 20,000 g in K24 refrigerated
centrifuged at 4°C. The supernatant was collected and the residue was reextracted with 1 ml of the acetate buffer and again centrifuged for 30 min. The supernatant were pooled together.

Gel filtration:

A sephadex G50 (superfine) column (1.2×64 cm) was used for the separation of neurohormones. The column was equilibrated with an ammonium acetate buffer (pH 8.5, 20mM). The clear supernatant was applied to the column and elution was carried out with a flow rate of 10 ml/hrs. Fraction of 1 ml was collected and the protein content and hyperglycemic activity of each fraction were determined.

Electrophoresis-

Polyacrylamide Gel electrophoresis (PAGE) was carried out in electrophoresis apparatus using a gel of 7.5% acrylamide at pH 8.9 (Tris-glycine buffer) as describe by Davis (1964). The thickness of gel was 15 mm and measured 18×18 cm. Sample obtained column showing maximum hyper glycemic activity (fraction no. 43-45) were dialysed against Tris-glycine buffer and were carefully applied with the help of micro syringe into different wells of the gel. Bromophenol blue was used as tracking dye.
Electrophoresis was carried out by applying a current of 12mA at 8°C until the tracking dye migrated 2-3 cm from the lower end of the gel. A portion of gel containing two wells used for ascertaining the electrophoretic mobility of the applied sample and the remaining major portion used to extract hyperglycemic activity. The gel was stained with coomassie brilliant blue R.0.025% in methanol /water/ acetic acid (227:227:46vv) and water 10:3:35,vv) the rest of the gel was cut transversely in to four segments (3 segment containing 3 band and fourth without band) based on localization of the band in the stained gel. Each segment of the gel was separately homogenized in 2 ml of chilled distilled water band centrifuged at 4°C for 10 min. The neurohormonal activity was tested in supernatant. Protein was determined by coomassie brilliant blue dye procedure (Spector 1978) with bovine serum albumin as a standard protein.

b) Toxicity Evaluation:

The stock solution of folicure pesticide was prepared by dissolving 1 ml of pesticide in 1000ml of tap water and from the stock solution different concentration solutions were prepared.
Snails were divided into several batches and each batch comprising of 10 snails. Each batch of snails was kept in plastic troughs containing one liter of water. The snails were exposed separately to different concentration of folicure pesticide and mortality rate was recorded after 24, 48, 72 and 96 hrs. of exposure. The \( \text{Lc}_{50} \) values were calculated for 24, 48, 72 and 96 hrs. by using the Finney Probit analysis chart (Finney 1971). The lethal concentration (\( \text{Lc}_{50} \)) values were calculated using critical analysis of regression line relating Probit and Log dose (Busvine 1971) (Table 1, Graph-1). The control mortality if it is appreciable will affect the precision of the results and a correction is usually applied by following formula.

\[
P_1 = \frac{p_0 - p_c}{100} \times p_c 	imes 100
\]

Where \( P_1 \) – Correction mortality

\( p_c \) – control mortality

\( p_0 \) – observed mortality

For calculation of the regression line relating probit and log dose “Evolution of toxicological statistics” (Busvine 1971) was strictly followed. Empirical probit values corresponding to the % mortality was located from probit analysis chart (Finney, 1971).
Table: 1

Calculation of log. Dose / Probit regression line for experiment in which snails Lymnea auricularia were exposed (24 hours) to different concentrations of folicure pesticide.

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Conc. of pesticide (ppm)</th>
<th>No. of animals used</th>
<th>% mortality kill</th>
<th>Log+2 Dose</th>
<th>Empiric Probit</th>
<th>Expecte d Probit</th>
<th>Weighing Coefficient</th>
<th>Weighing</th>
<th>W</th>
<th>wx</th>
<th>wx²</th>
<th>wy</th>
<th>wy²</th>
<th>wxy</th>
<th>y’</th>
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<tr>
<td>1</td>
<td>0.075</td>
<td>10</td>
<td>10</td>
<td>0.875</td>
<td>3.72</td>
<td>3.21</td>
<td>4.007</td>
<td>0.180</td>
<td>1.80</td>
<td>1.575</td>
<td>1.378</td>
<td>7.213</td>
<td>28.901</td>
<td>6.312</td>
<td>3.46</td>
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<tr>
<td>2</td>
<td>0.15</td>
<td>10</td>
<td>20</td>
<td>1.176</td>
<td>4.16</td>
<td>4.02</td>
<td>4.166</td>
<td>0.439</td>
<td>4.39</td>
<td>5.163</td>
<td>6.078</td>
<td>18.29</td>
<td>76.191</td>
<td>21.50</td>
<td>4.16</td>
</tr>
<tr>
<td>3</td>
<td>0.225</td>
<td>10</td>
<td>30</td>
<td>1.352</td>
<td>4.48</td>
<td>4.48</td>
<td>4.480</td>
<td>0.558</td>
<td>5.58</td>
<td>7.545</td>
<td>10.20</td>
<td>24.99</td>
<td>11.993</td>
<td>33.80</td>
<td>4.57</td>
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<td>10</td>
<td>40</td>
<td>1.477</td>
<td>4.75</td>
<td>4.80</td>
<td>4.744</td>
<td>0.627</td>
<td>6.27</td>
<td>9.264</td>
<td>13.68</td>
<td>29.74</td>
<td>141.11</td>
<td>43.93</td>
<td>4.86</td>
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<tr>
<td>5</td>
<td>0.375</td>
<td>10</td>
<td>50</td>
<td>1.574</td>
<td>5.00</td>
<td>5.05</td>
<td>5.005</td>
<td>0.637</td>
<td>6.37</td>
<td>10.02</td>
<td>15.78</td>
<td>31.88</td>
<td>159.56</td>
<td>50.18</td>
<td>5.09</td>
</tr>
<tr>
<td>6</td>
<td>0.45</td>
<td>10</td>
<td>60</td>
<td>1.653</td>
<td>5.25</td>
<td>5.25</td>
<td>5.256</td>
<td>0.627</td>
<td>6.27</td>
<td>10.36</td>
<td>17.13</td>
<td>32.95</td>
<td>173.21</td>
<td>54.48</td>
<td>5.27</td>
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<tr>
<td>7</td>
<td>0.525</td>
<td>10</td>
<td>60</td>
<td>1.720</td>
<td>5.25</td>
<td>5.45</td>
<td>5.252</td>
<td>0.601</td>
<td>6.01</td>
<td>10.33</td>
<td>17.78</td>
<td>31.56</td>
<td>165.77</td>
<td>54.29</td>
<td>5.43</td>
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<tr>
<td>8</td>
<td>0.60</td>
<td>10</td>
<td>70</td>
<td>1.778</td>
<td>5.52</td>
<td>5.60</td>
<td>5.520</td>
<td>0.558</td>
<td>5.58</td>
<td>9.922</td>
<td>17.64</td>
<td>30.80</td>
<td>170.02</td>
<td>54.77</td>
<td>5.56</td>
</tr>
<tr>
<td>9</td>
<td>0.70</td>
<td>10</td>
<td>80</td>
<td>1.845</td>
<td>5.84</td>
<td>5.84</td>
<td>5.840</td>
<td>0.503</td>
<td>5.03</td>
<td>9.280</td>
<td>17.12</td>
<td>29.37</td>
<td>171.55</td>
<td>54.20</td>
<td>5.72</td>
</tr>
<tr>
<td>10</td>
<td>0.80</td>
<td>10</td>
<td>90</td>
<td>1.903</td>
<td>6.28</td>
<td>5.94</td>
<td>6.214</td>
<td>0.471</td>
<td>4.71</td>
<td>8.964</td>
<td>17.05</td>
<td>29.26</td>
<td>181.87</td>
<td>55.70</td>
<td>5.85</td>
</tr>
</tbody>
</table>

Σw=52.01 Σwx=82.42 Σwx²=133.868 Σwy=266.090
Σwy²=1380.198

Σwxy=429.192
Regression line
Provisional line
24 hrs. Lc 50 Value = 0.3 ppm
Results
Results:

The environmental parameters like temperature-24°C, pH 6.8, Dissolved Oxygen 4.8 mg/L and total hardness 180 mg/L was maintained throughout the year.

a) The elution of cerebral ganglion extract on sephadex G-50 column was resolved in to four different protein peak (Graph-2) Hyperglycemic activity was observed in second peak (fraction no. 41-47) while peak first (fraction no.16-40). Peak third (fraction no.48- 55) and peak four (fraction no.59-68) did not show any hyperglycemic hormone activity. It suggests that 1st peak corresponds to high molecular weight protein while peak 3rd and 4th contain low molecular weight protein as compared to hyperglycemic hormone.

b) The Lc₅₀ values of fresh water snail *Lymnea auricularia* exposed to folicure pesticide were calculated. The final regression equation for 24 hrs. to 96 hrs . shown in Table -2.

The result of present investigation reveals that % mortality increase progressively up to 96 hrs. The Lc₅₀ values decrease with increase in the exposure period showed an inverse relation.
The Chromatographic activity of each fraction was tested on 10 animals/fraction and protein content is represented in PI, PII, PIII & PIV represent various protein peaks.
Table -2

Relative toxicity of folicure pesticide when fresh water snail *Lymnea auricularia* exposed to acute concentration (24 hrs. to 96 hrs.)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Exposure period</th>
<th>Lc$_{50}$ values +SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24 hrs.</td>
<td>0.35 ± 0.006</td>
</tr>
<tr>
<td>2</td>
<td>48 hrs.</td>
<td>0.25 ± 0.004</td>
</tr>
<tr>
<td>3</td>
<td>72 hrs.</td>
<td>0.20 ± 0.0016</td>
</tr>
<tr>
<td>4</td>
<td>96 hrs.</td>
<td>0.14 ± 0.0012</td>
</tr>
</tbody>
</table>
24 hrs. Lc 50 Value = 0.3548 ppm

Graph 3

Probit

Log Conc. + 2
48 hrs. Lc 50 Value = 0.253

Log Conc. + 2
Graph 4
72 hrs. Lc 50 Value = 0.2041

Graph 5

1.31 - 2 = 0.2041
96 hrs. LC 50 Value = 0.1445

Graph 6
The LC$_{50}$ Values of folicure pesticide to the snail *Lymnea auricularia* for 24, 48, 72 and 96 hrs. were found to be 0.35, 0.25, 0.20 and 0.14 ppm respectively (graph3,4,5,6).

The present experiment revels that the % mortality increases with increase in concentration of pesticide and exposure period.
Discussion
Discussion:

The molluscan endocrine centers are the master center known to regulate the physiological functions of the animals. Pollutants may directly act on the neuroendocrine centers which governs the physiological functions. In molluscan various physiological processes like respiration, digestion, metamorphosis and reproduction are under hormonal control. Several hormones are produced by the neurosecretory cells. Any stress either pollutional or any other type is known to manifest a disorder in the functioning of neuroendocrine centers of the animals.

In the present investigation it was observed that most of the neuroendocrine hormones are proteinious in nature. Similar observations were made by Nagabhushanam and Vasanta (1971) on fresh water caridian prawn *Cardina weberi*. Nagabhushanam et.al. (1989) studied the proteineous nature of hyperglycemic hormone on fresh water crab *Barytelphusa cunicularis*. Isolation and characterization of neurohormones were done by Vreugdenhil et.al. (1988) on fresh water snail *Lymnaca stagnalis*. Likw et.al. (1997) reported that neuroendocrine light green cells (LGCS) control body growth and metabolism of the fresh water snail *Lymnea stagnalis*. Chiu et. al. (1979) reported the purification and primary
structure of the neuropeptides egg laying hormones of *Aplysia californica*. Ebberink (1985) purified and amino acid sequence of the ovulation neurohormones of fresh water snail *Lymnea stagnalis* further they reported that this hormone is stored and released at the periphery of the intercerebral commissure. Eliassen et.al. (1991) studied the Isolation and characterization of neuropeptides that mimic prolonged inhibition produced by all neurons in *Aplysia*. Mahmud et.al.(2008) reported the characterization of neurons in the visceral ganglia of the green tipped mussel *Perna canaliculus* using antibodies raised against neuropeptides and neurotransmitters.

In the present investigation when fresh water snail *Lymnea auricularia* exposed to folicure pesticide it showed qualitative as well as quantitative changes in the neurosecretory cells of the snail. During acute and chronic exposure to folicure pesticide, vacuolization, necrosis of neurosecretory cells and loss in compactness of neurosecretory cells were observed. Chandrakala Patil et.al. (2008) studied the effect of pesticide monochrotophos on neuroendocrine regulation of fresh water crab *Barytelphusa gurini*. Further they reported that due to pesticidal exposure drastic changes in neurosecretory cells were observed.
From the last few decades fresh water environment has become highly polluted throughout the biosphere with various pesticides which have become essential and virtually irreplaceable tool of the agriculturists to increase the production of food, production of wood and controlling the dreadful diseases. In addition to this, the industrial wastes and runoff water from agricultural land causes the contamination of aquatic ecosystem.

The result of the present investigation reveals that acute toxicity tests (24 hrs. to 96 hrs.) shows a relationship between the length of exposure and concentration of pesticide. Acute toxicity involves the damage to the organisms by the fastest acting mechanism. The toxicity test is the determination of relative sensitivity of various species to pollutant. Similar observations were made by Sambashiva rao et.al. (1990) on marine edible crab *Scylla serrata* exposed to the pesticide endocel. Chaudhari et.al. (1988) reported many behavioral changes in pesticide exposed snail *Bellamya bengelensis* like sudden withdrawal of foot inside the shell, closing of operculum and mucous secretion. Martin et. al.(1990) studied the toxic effect of methyl parathion on fresh water prawn *caridina weberi*, Nagabhushanam et.al. (1991) reported the toxicity of copper sulphate and zinc sulphate on fresh water crab *Barytelphusa guerini*. Yadav et.al. (1990)
studied the behavioral assessment of pesticide endosulfan on fresh water prawn *caridina weberi*. Fargosova (1997) reported the long term toxicity value of pesticide on *Daphnia magna*. Sujata et.al. (1996) investigated the sublethal effects of TBT oxides exposure using tropical estuarine clam *Villorita cyprenoides*. Cheng and Chen (2002) studied the toxicity of nitrite on fresh water prawn *Macrobrachium rosenbergi*. Kungolos et.al. (2004) studied the toxicity properties of four organotin compounds and heavy metals on the fresh water crustaceans *Daphnia magna*. Yadav and Mehevi (2004) reported the toxicity of fenervate pesticides to fresh water crab *Barytelphusa cunicularis*. Keshavan et.al.(2005) has studied the uncoordinated movement in fresh water crab *Barytelphusa guerini* exposed to hilden pesticide.

Kharat et.al. (2009) showed the Lc$_{50}$ for 48 hrs. of organotin tributyltin chloride on the rate of oxygen consumption of fresh water prawn *Macrobranchium kistenensis* has been determined. Shejule et.al.(2006) has reported the toxicity of TBTO on fresh water prawn *Macrobrachium kistnensis* and further reported that Lc$_{50}$ values decreased with increasing exposure period. Kulkarni et.al.(2005) studied the toxic effect of Hilden pesticide on fresh water mussel *Lamellidens corrianus*. 
Pardeshi et al. (2007) studied acute and chronic toxicity of mercuric chloride on Parreysia cylindrica. Agoes et al. (2008) studied the toxicity of drilling waste and its impact on gill structure of tiger prawns Penaeus monodon. Further they reported that the formation of mucus film over the body surface and gills which interferes with the respiratory function. Seker et al. (2009) reported the toxic effect of textile dyes on fresh water field crab Spiralothenphusa hydrodroma and further reported that cause of death in crustaceans after chromium exposure may be due to damages of respiratory surfaces. Yadav (2010) reported the toxicity of metasystox pesticide to fresh water crab Macrobrachium lammurii.

From the above finding it can be concluded that the toxicity of pesticide folicure in water is influenced by other interacting factors and physiological state of the animal.