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Study of the toxicity of two pharmaceuticals on invertebrate Hydra Vulgaris and observing effects on morphology and physiology

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ABSTRACT

The Cnidarian Hydra have been extensively used as a model organism studying the teratogenic and toxic potential of numerous toxins throughout the years and are more recently growing in popularity to assess the impacts of environmental pollutants. Hydra are an appropriate bioindicator species for use in environmental assessment owing to their easily measurable physical (morphology), biochemical (xenobiotic biotransformation; oxidative stress), behavioural (feeding) and reproductive (sexual and asexual) endpoints. Pharmaceuticals enter natural waters through sewage effluent and landfills and present an unknown risk to aquatic species including freshwater invertebrates. The cnidarian Hydra has been widely used to assess the acute toxicity of freshwater pollutants, but very little is known about pollutant accumulation by this animal. This study was conducted to study the effect of commonly used drugs, Paracetamol and Acetyl salicylic acid (Aspirin) on the morphological and physiological working of Hydra vulgaris. The Hydra were exposed to 4 different concentrations of each drug for 24 hour and then observed for morphological and physiological changes.

Keywords— *H. vulgaris, Paracetamol, Aspirin, Waste Water, Toxicity*

1. INTRODUCTION

Hydra vulgaris is a small animal freshwater hydroid with length from 10mm to 30mm and width about 1 mm belonging to phylum Cnidaria. Cnidarians are distinguished from all other animals by having cnidocytes that fire harpoon like structures and are usually used mainly to capture prey or as an anchor in some sedentary cnidarians. Cnidarians are also distinguished by the fact that they have only one opening in their body for ingestion and excretion i.e. they don't have a separate mouth and anus. Hydra belongs to class Hydrozoa and family Hydridae. Hydra are native to the temperate and tropical regions.

Fig 1: Hydra attached to substrate

H. vulgaris have four to twelve tentacles that protrude from just outside the mouth. They feed by extending their tentacles and waiting for food to touch the tentacles. Each tentacle, or cnida (plural: cnidae), is clothed with highly specialized stinging cells called cnidocytes. Cnidocytes contain specialized structures called nematocysts, which look like miniature light bulbs with a coiled thread inside. Upon contact with prey, the contents of the nematocyst are explosively discharged, firing a dart-like thread containing neurotoxins into whatever triggered the release. This can paralyze the prey, especially if many hundreds of nematocysts are fired.

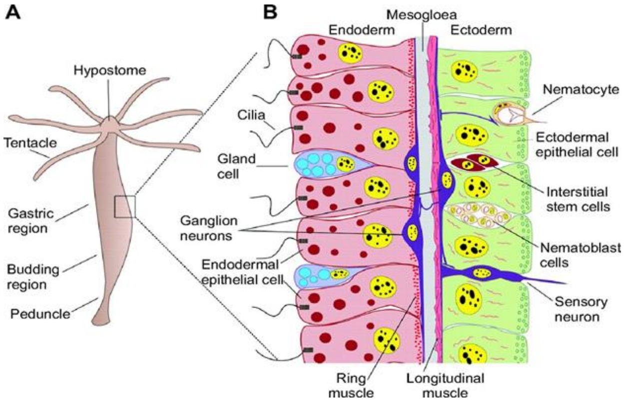

Fig. 2: General anatomy and cells of Hydra

Hydra reproduce asexually by producing buds in the body wall, which grow to be miniature adults and break away when they are mature. Several scientists have had curiosity in Hydra for years due to three qualities such as, its regenerative qualities, its apparent property of not undergoing senescence or biological aging and its sensitivity to a number of different teratogenic compounds as well as the toxicity of potential toxic compounds.

In recent times, scientists are using Hydra to assess the impacts of environmental pollutants as they are appropriate bio-indicator species for use in environmental assessment owing to their easily measurable physical (morphology), biochemical, behavioral (feeding) and reproductive (sexual and asexual) endpoints. Hydra have been used to assess the environmental impacts of numerous environmental pollutants including metals, organic toxicants including pharmaceuticals and endocrine disrupting compounds, nanomaterials and industrial and municipal effluents. They have been found to be among the most sensitive animals tested for metals and certain effluents, comparing favorably with more standardized toxicity tests. Hydra have been extensively used and are regarded as a model organism in aquatic toxicology.

A medication is a substance that is taken into or placed on the body that are used to cure a disease or condition or to treat a medical condition. Pharmaceuticals are designed to interact with biological systems in order to bring about beneficial effects principally in man but also in domestic and farm animals. Many of the pharmaceuticals applied in human medical care are not completely eliminated by the body, often excreted only slightly transformed or even unchanged. It is important that we understand the extent to which invertebrates may be affected by the presence of active pharmaceutical agents. The drugs used in the experiment are 2 of the most prescribed drugs.

Paracetamol also known as acetaminophen and APAP, is a medication used to treat pain and fever. It is typically used for mild to moderate pain relief. Paracetamol is used to treat many conditions such as headache, muscle aches, arthritis, backache, toothaches, colds, and fevers.

Fig. 3: Structure of Paracetamol

Also known as Aspirin, acetylsalicylic acid is a commonly used drug for the treatment of pain and fever due to various causes. Acetylsalicylic acid has both anti-inflammatory and antipyretic effects. This drug also inhibits platelet aggregation and is used in the prevention of blood clots stroke, and myocardial infarction.

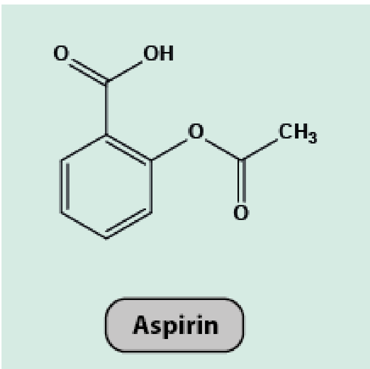

Fig. 4: Structure of Aspirin

2. REVIEW OF LITERATURE

(a) P. David, K. Wanchamai, M. Carsten- Acute toxicity was based on the established technique looking at morphological changes in the Hydra, while recently developed endpoints of feeding behaviour, attachment and growth (hydranth number) were used to measure chronic effects..

(b) Quinn. B, Gagne. F, Blaise. C - Hydra have been used to assess the environmental impacts of numerous environmental pollutants including metals, organic toxicants (including pharmaceuticals and endocrine disrupting compounds), nanomaterials and industrial and municipal effluents. They have been found to be among the most sensitive animals tested for metals and certain effluents, comparing favourably with more standardised toxicity tests.

(c) Quinn. B, Gagne. F, Blaise. C-Pharmaceuticals are biologically potent chemicals interacting with specific drug targets at low concentrations. Many of these drug targets are conserved across various organisms. Consequently, even the low environmental levels arising from the usage of certain drugs may result in adverse effects on wildlife.

(d) Daughton, G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Diagnostic agents, "nutraceuticals," fragrances, sun-screen agents, and numerous others. These compounds and their bioactive metabolites can be continually introduced to the aquatic environment as complex mixtures via a number of routes but primarily by both untreated and treated sewage.

(e) Halling-Sørensen, B., Nielsen, N., Lansky, P.F., Ingerslev, F., Lutzhøft Jørgensen, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment. This paper outlines the different anticipated exposure routes to the environment, surnmarises the legislation on the subject and gives an outline of present knowledge of occurrence, fate and effect on both the aquatic and terrestrial environments of medical substances.

(f) Wanchamai Karntanut, David Pascoe-A comparison of methods for measuring acute toxicity to Hydra vulgaris - l. The aim of the present investigation is to compare the conventional measure of acute toxicity with that obtained by measuring progressive changes in animal morphology using copper, cadmium and zinc.

(g) Howard M. Lenhoff -Culturing Large Numbers of Hydra – Lenhoff described two methods for culturing large numbers of hydra. The first, the tray method requires no specially constructed equipment. It allows a person with a little experience to culture hundreds of thousands of animals daily with relatively little effort. It is the method used by most investigaters.

(h) Patricia Bossert, Brigitte Galliot- How to use Hydra as a model system to teach biology in the classroom- (Cell Biology)-all basic cell types shared by eumetazoans can be found in Hydra, an analysis of the various cell types found in Hydra, i.e. stem cells, epithelial cells, neurons, gland cells, germ cells.

(i) O.K Wilby , J.M Tesh- Hydra assay as an early screen for teratogenic potential - The current study was designed to investigate the sensitivity of the HRA in assessing the teratogenic potential of a series of related molecules, that is six retinoids of known mammalian teratogenic potential.

(j) Susan Jobling , John P. Sumpter , David Sheahan , Julia A. Osborne , Peter Matthiessen- Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkyl phenolic chemicals- Exposure of male rainbow trout to four different alkyl phenolic chemicals caused synthesis of vitellogenin, a process normally dependent on endogenous estrogens, and a concomitant inhibition of testicular growth.

(k) Barbara Benson and G. M. Boush- Effect of Pesticides and PCBs on Budding Rates of Green Hydra - The evidence regarding the impact of these chemicals on aquatic ecosystems consists mainly of acute toxicity data. However, subtle, sub lethal effects, especially on lower invertebrates, have generally been neglected, although such effects must be both common and important and play a vital role in the overall impact of a toxicant on the environment.

(l) Christian Blaise Takashi Kusui - Acute Toxicity Assessment of Industrial Effluents with a Microplate-Based Hydra assay - The acute toxicity potential of ten industrial plants located in Toyama Prefecture (Japan) was appraised with a microplate-based assay developed with the freshwater cnidarian Hydra attenuata.

(m) Brun GL1, Bernier M, Losier R, Doe K, Jackman P, Lee HB- Pharmaceutically active compounds in Atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity- Ten acidic and two neutral pharmaceuticals were detected in the effluents of eight sewage treatment plants (STPs) from across Atlantic Canada. Concentrations varied between nondetectable and 35 microg/L. The analgesic, nonsteroidal anti-inflammatory drugs ibuprofen and naproxen were predominant. Bioassays assessing acute and chronic effects on four organisms were conducted on four high-use drugs: Acetaminophen, ibuprofen, naproxen, and salicylic acid (metabolite of acetyl salicylic acid). Results indicated no negative effects except for the chronic algal (Selanastrum capricornutum) growth test on ibuprofen (no-observed-effect concentration,

(n) Mark Crane, Chris Watts, Tatiana Boucard- Chronic aquatic environmental risks from exposure to human pharmaceuticals- Maximum concentrations ethinyloestradiolhave been measured so biological effects on invertebrates at low ng l^{-1} levels are environmentally relevant. Other estrogens and the androgen methyl testosterone also cause effects at very low concentrations, and a wide range of other therapeutic classes cause effects at concentrations.

(o) M.D. Hernando , M. Mezcua , A.R. Fernandez-Alba , D. Barcelo´-Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments - The increasing attention, on pharmaceutical residues as potential pollutants, is due that they often have similar physico-chemical behaviour than other harmful xenobiotics which are persistent or produce adverse effects. In addition, by contrast with regulated pollutants, which often have longer environmental half-lives, its continuous introduction in the environment may make them "pseudopersistents"

(p) S. Pachura-Bouchet, C. Blaise, P. Vasseur-Toxicity of Nonylphenol on the Cnidarian Hydra attenuata and Environmental Risk Assessment -. Nonylphenol (NP) is a critical APE metabolite because of its recalcitrance to biodegradation, toxicity, and ability to bio-accumulate in aquatic organisms. Studies of NP effects in vertebrates demonstrated estrogenic disrupting properties in fish, birds, reptiles, and mammal cells in which NP displaces the natural estrogen from its receptor. Results showed that hydra appeared as one of the most sensitive species to acute and chronic toxicity of NP compared to several freshwater invertebrates. Regeneration was disrupted at NP concentrations lower than those affecting survival.

(q) Douglas A. Holdway, Katrina Lok, Michael Semaan-The Acute and Chronic Toxicity of Cadmium and Zinc to Two Hydra Species - Results showed that both the hydra species were more sensitive to cadmium than to zinc, and that green hydra were more sensitive than pink Hydra. Green hydra appeared to be excellent freshwater invertebrate models for testing dissolved metals based on their sensitivity and the ability to rapidly assess population reproduction in the laboratory.

(r) Galliot B, Miljkovic-Licina M, de Rosa R, Chera S. - Hydra, a niche for cell and developmental plasticity- The silencing of genes whose expression is restricted to specific cell types and/or specific regeneration stages opens avenues to decipher the molecular control of the cellular plasticity underlying head regeneration in hydra. In this review, we highlight recent studies that identified genes involved in the immediate cytoprotective function played by gland cells after amputation; the early dedifferentiation of digestive cells into blastema-like cells during head regeneration, and the early late proliferation of neuronal progenitors required for head patterning

(s) A. R. D. Stebbing and A. J. Pomroy - A sublethal technique for assessing the effects of contaminants using hydra littoralis - A technique is described for estimating the biological effects of low levels of contaminants using as an index of response the rate of asexual reproduction of Hydra littoralis. Thresholds of sensitivity are low enough to show that Hydra would respond to copper levels found in contaminated waters and could provide an index of biological quality of water samples in the laboratory.

(t) Carmel A. Pollino and Douglas A. Holdway- Potential of Two Hydra Species as Standard Toxicity Test Animals - the potential for using pink hydra (Hydra vulgaris) and green hydra (Hydra viridissima) as a model invertebrate for the toxicity testing of xenobiotics was investigated. Test compounds were 4-chlorophenol, endosulfan, and copper. Results indicate that hydra have the potential for use in acute and subchronic toxicity testing of inorganic toxicants, but have a low sensitivity to organic toxicants.

3. METHODOLOGY

3.1 Preparation of culture for the test organisms

Hydra vulgaris were cultured in Hydra medium at 21± 1 °C in glass bowl.

Fig. 5: Hydra is glass bowl

Culture medium (100X) was composed using the following:
a.KCl - 0.1 mM – 0.074 gm
b. NaCl – 1 mM - 0.58 gm
c.$MgSO_4.7H_2O$ – 0.1 mM- 0.24 gm
d. $CaCl_2.2H_2O$ – 1mM – 1.1 gm
e.Tris base- 1mM- 1.21 gm
All the salts were dissolved in 100 ml distilled water and autoclaved for 40 mins.
The pH was adjusted at 8 and 100 x medium was stored in a glass bottle.
For the Hydra culture, 100X media was diluted to 1x using autoclaved distilled water.
Hydra were transferred in new 1x Hydra medium after 24h.
Hydra were fed regularly with Artemia larvae hatched in salt water overnight.


Fig. 6, 7 & 8: Workstation where experiment was conducted, beaker containing Artemia larvae in salt water, bottles of Artemia egg and rock salt

3.2 Preparation of test chemicals

The two drugs selected for the experiment were Paracetamol and Aspirin. Both the drugs were obtained in their pure forms from wholesale drugs distributor located in Pune city. 100ml stock solutions were prepared for both the drugs. 1.5 gm of powdered Paracetamol was mixed in 100ml distilled water and 1.8 gm of Aspirin in 100 ml distilled water.

Table 1: Concentration of stock solutions used

Sr. No	Aspirin (µl)	Paracetamol (µl)
1.	0.5	0.5
2.	0.3	0.3
3.	0.1	0.1
4.	0.05	0.05

3.3 Testing for acute toxicity

Toxicity testing was done by transferring 8-10 non-budding Hydra to a small glass cavity block containing 5ml of Hydra medium (1x). To this different concentrations of the stocks of the drugs were added (0.05, 0.1, 0.3, 0.5ml) and the Hydra were kept in the solutions for 24h. Toxicity was assessed by microscopically recording the morphological status of each polyp each day and assigning a score from 10 (normal) to 0 (disintegrated) according to a table devised by Wilby in 1988. *[Table 2]

3.4 Preparation of slides for observation of Hydra cells

A small secondary procedure was carried out dealing with separation of cells from Hydra and observing the cells under a light microscope. 5ml of maceration fluid containing glycerol and acetic acid in the exact ratio 1:1 was prepared. 2ml of pure SDS solution was prepared. 50 µl of maceration fluid was taken in a 1.5 ml autoclaved eppendorf tube to which 5-6 Hydra were added and the eppendorf tube was lightly mixed. The eppendorf tube was then incubated at room temperature for 25-30 minutes. After 30 minutes, 1-2 drops of SDS solution were added to the Eppendorf and lightly mixed. The contents were spread on glass slide and left to dry overnight. The slides were then stained using two different stains namely methylene blue and toluidine blue for 5-10 minutes. Stained slides were then observed under a light microscope at 40x magnification and various cells were identified.

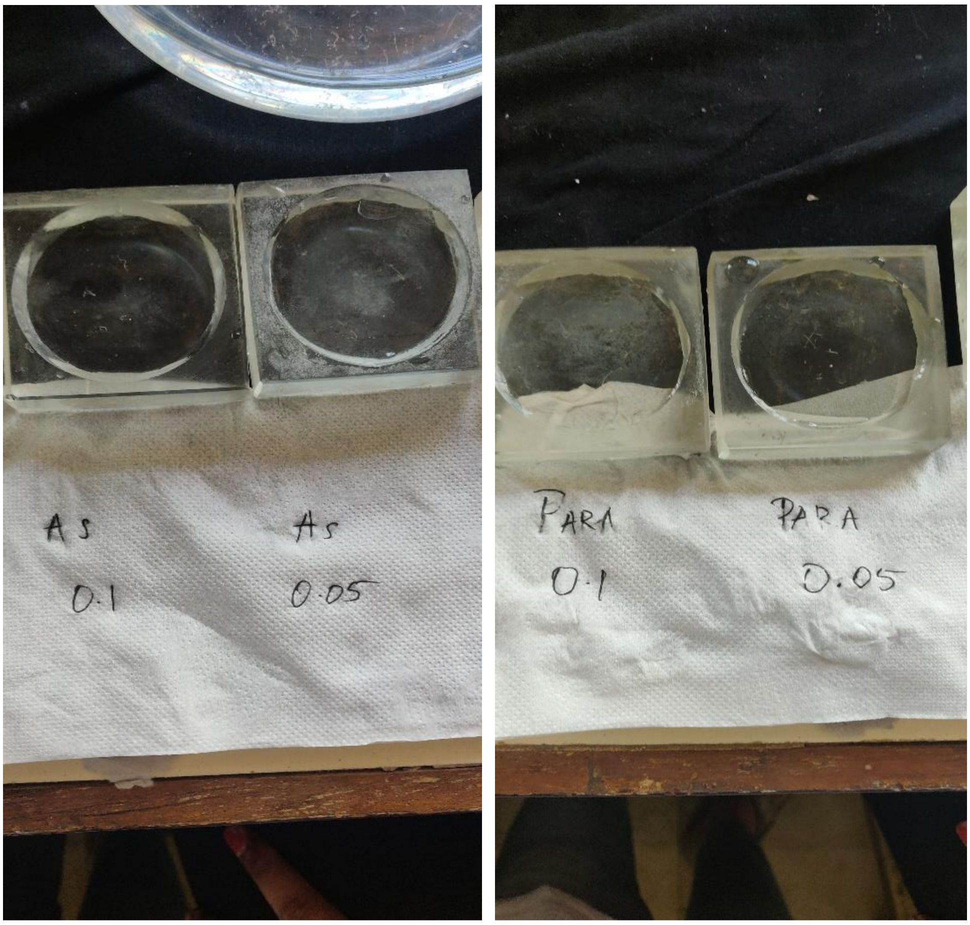

Fig. 9, 10 & 11: Set up of experiment with Hydra in different stock solutions, Hydra in cavity blocks with Aspirin and Paracetamol solutions

3.5 Effect of temperature on *Hydra vulgaris*

- It does not need any special requirement or method.
- Just put the *Hydra* containing bowl at room temperature.
- Observe the result after 24 hours.

4. RESULTS

All the Hydra survived in the test solutions for 48 hours and median toxicity scores indicating the effects of each drug to Hydra were compared to the key table assessing the acute toxicity effects on Hydra.

Table 1: Key for assessing progressive toxic effects in Hydra polyps (Wilby 1988)

Score	Morphology of polyp
10	Extended tentacles and body reactive
9	Partially contracted, slow reactions
8	Clubbed tentacles, body slightly contracted
7	Shortened tentacles, body slightly contracted
6	Tentacles and body shortened
5	Totally contracted, tentacles visible
4	Totally contracted, no visible tentacles
3	Expanded, tentacles visible
2	Expanded, no visible tentacles
1	Dead but intact
0	Disintegrated

4.1 (a) Observed Results in Hydra placed in Aspirin:

Table 2: Concentration of stock, morphological differences seen in Hydra & toxicity score

Concentration 0f Aspirin	Morphological Changes observed	Acute toxicity score
0.05µg	Body slightly contracted but tentacles are extended and active.	9
0.1µg	Body contracted with clubbed tentacles, Hydra become less active.	7
0.3µg	Body contracted with clubbed tentacles, Hydra become less active.	7
0.5µg	Body contracted with highly clubbed tentacles.	≤ 7

Based on observation of morphology and physiology of Hydra in Aspirin test solution, it is seen that even at the highest concentration of 0.5 µg there is not a very big impact on the invertebrate. At 0.05 µg, the hydra showed close to normal morphology with slight contraction of the body and extended tentacles. The hydra was fed with Artemia after 24 hours and all the Hydra in the test sample show normal feeding habits. At 0.1 µg and at 0.3 µg the body of Hydra slightly contracts. The tentacles contract and become clubbed. The hydra become comparatively less active and showed erratic feeding, that is not all Hydra fed on the Artemia only a few selected Hydra showed feeding. At 0.5 µg however the Hydra showed maximum morphological and physiological changes, the body was contracted and the tentacles were highly clubbed. The hydra showed close to no activity along with complete inhibition of feeding.

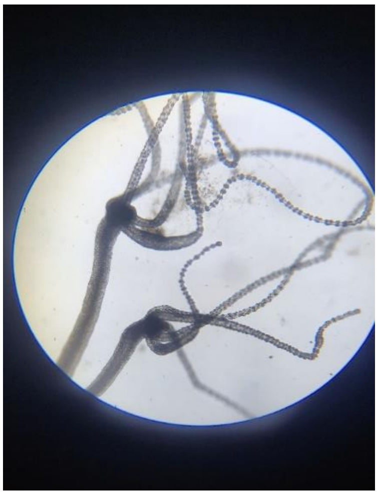

Fig. 12: Normal Hydra under light microscope

Fig. 13: Hydra in 0.05 concentration

Fig. 14: Hydra in 0.1 concentration

Fig. 15: Hydra in 0.3 concentration

Fig. 16: Hydra in 0.5 concentration

4.1 (b) Observed Results of Hydra placed in Paracetamol

Table 4: Concentration of stock, morphological differences observed and toxicity score.

Concentration of Paracetamol	Morphological Changes Observation	Acute Toxicity Score
0.05	Body is extended along with tentacles and Hydra shows normal activity.	10
0.1	Body and tentacles both become contracted and activity reduces greatly.	7
0.3	Body becomes totally contracted along with tentacles becoming short. No activity is seen.	5
0.5	Body is totally contracted. Tentacles are no more visible and no activity.	4

At 0.05 concentration of Paracetamol test solution, the Hydra show normal morphology and physiology. The body remains mostly elongate and tentacles are extended and active. They show normal feeding. At 0.1 the body and tentacles become contracted. Activity also reduces. Feeding is erratic with only a few Hydra feeding on Artemia. At 0.3 concentration, the body of Hydra becomes highly contracted. Tentacles become greatly shortened. The body shows no activity and no feeding. At 0.5, the body is highly contracted and tentacles are no longer visible. The hydra almost appears like a ball. Activity is at halt and feeding is absent.

Fig. 17: Hydra under electron microscope

Fig. 18: Hydra in 0.05 concentration

Fig. 19: Hydra in 0.1 concentration

Fig. 20: Hydra in 0.3 concentration

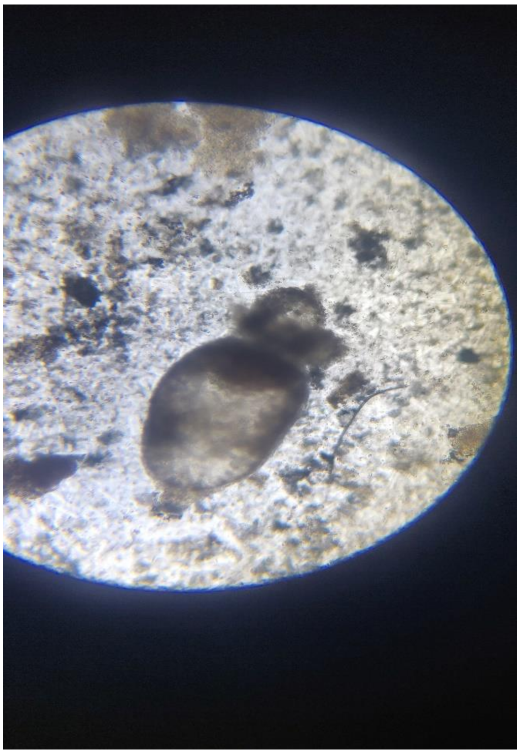

Fig. 21: Hydra under 0.5 concentration

4.2 Identification of different cell types

There are several types of cells were observed under the microscope.

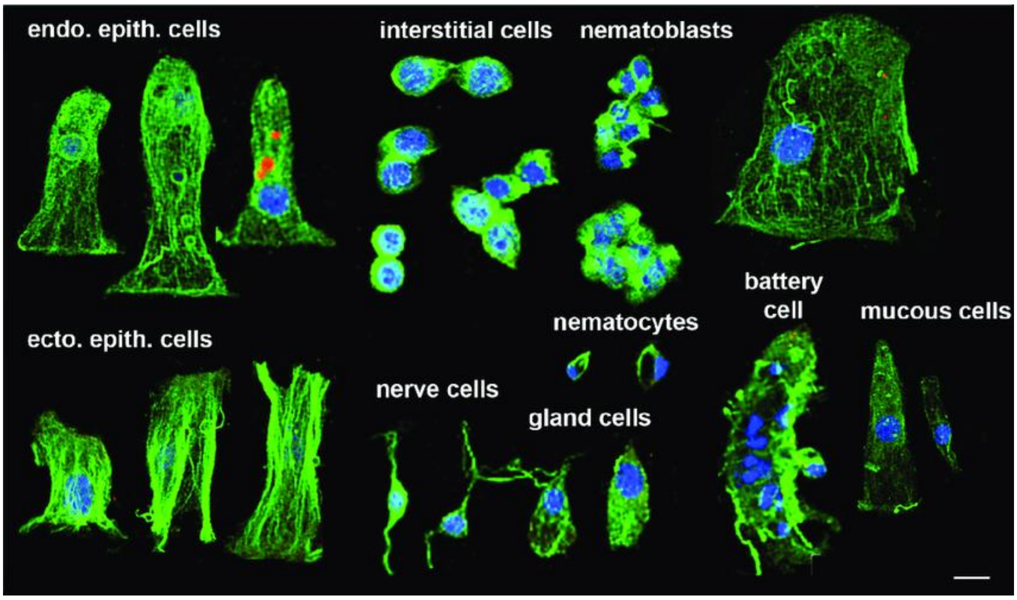

Fig. 22: Previously identified cells of Hydra

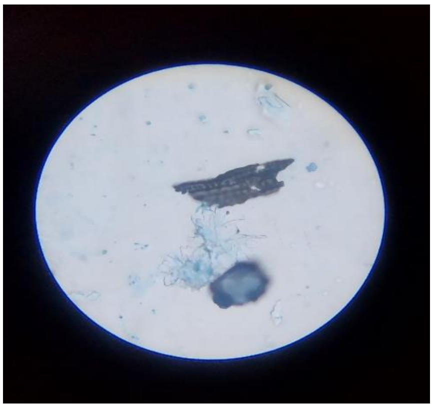

Fig. 23: Ecto epithelial cell

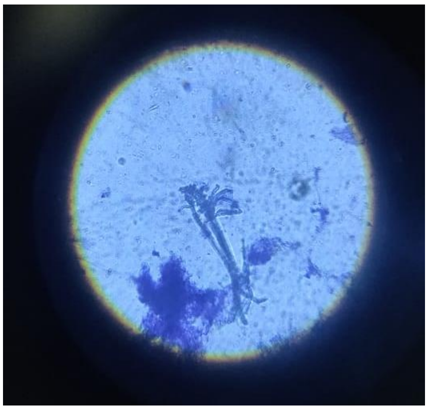

Fig. 24: Ecto epithelial cell

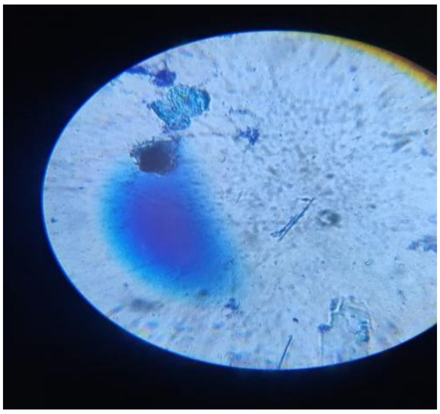

Fig. 26: Nematoblast

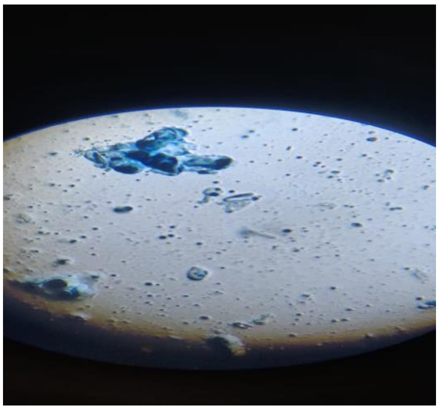

Fig. 27

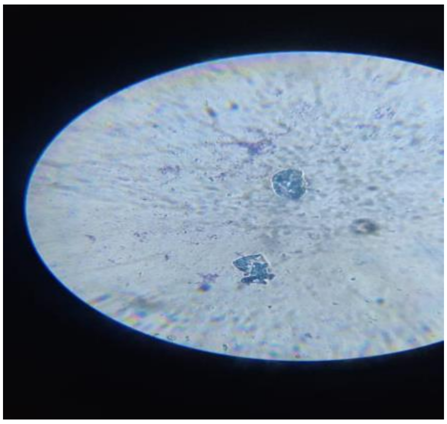

Fig. 28: Nematocyte

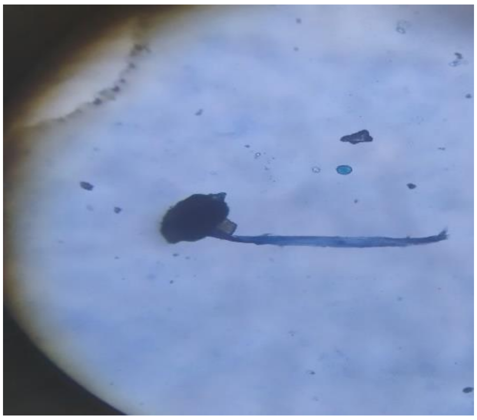

Fig. 29: Nerve cells

4.3 Effect of temperature on Hydra

Hydra was left in uncontrolled temperature conditions for 24 h and it was observed that majority of hydra disintegrated and dead.

5. DISCUSSION

Toxicity in Hydra is typically measured by drastic changes in morphology assessed using a binocular microscope. As the amount of toxicant increases Hydra progressively exhibit morphological changes that were conventionally measured and expressed based on the observed changes in the animal with normal, bulbed or clubbed tentacles, shortened tentacles, tulip phase and disintegration as endpoints.

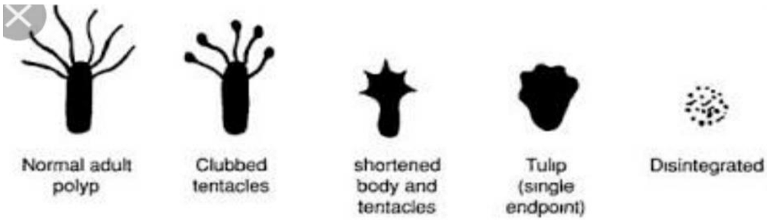

Fig. 30: Morphological changes sequence

The Hydra body column is formed of two walls of myoepithelial cells, the endoderm and the ectoderm separated by an extracellular layer named mesoglea. These two types of myoepithelial cells can easily be distinguished on macerated tissues, as the former are rather cubic when the latter are elongated with a clear apico-basal polarity. The central part of the animal contains an excess of stem cells compared to the extremities where cells stop dividing and terminally differentiate. A single hydra is composed of 50,000 to 100,000 cells which consist several types of cells like nerve cells, endo. epith cells, ecto.epith cells, nematocytes, nematoblasts etc

Pharmaceuticals differ from other pollutants entering water in two key respects (i) they are actually designed to have effects upon biological systems by modification of physiological/biochemical function, and (ii) unlike many other pollutants, e.g. agrochemicals, which are discharged or released sporadically, pharmaceuticals are continuously introduced into surface waters causing lifecycle exposures of the biota.

Although there are several routes for pharmaceuticals to enter the aquatic environment including the application of various drugs in farming and aquaculture, leachate from landfill and effluent from hospitals, waste water effluent is known to be the most significant of these.

Little is known about the chronic effects of these compounds on non-target species, particularly invertebrates.
As we discussed earlier the Hydra require the 21±1°C for their growth and survival but the temperature get increased i.e 37°C then the activity of hydra get decreased and they die.

6. CONCLUSION

Hydra are used for the biological toxicity testing of chemicals containing waste water using freshwater organisms. The part of success of the use of Hydra in aquatic toxicology is due to the wide array of lethal and non-lethal endpoints that can be used in both acute and chronic studies.

It is generally thought that the aquatic environment is particularly at risk to pollution by pharmaceuticals due to their continued introduction into surface waters from waste water treatment plants. Although it is unlikely that these pollutants will be found at concentrations high enough to illicit an acute effect, evidence is mounting to suggest that they may be present in sufficient concentrations to cause chronic effects.

The results obtained from the performed experiment showed that the pharmaceuticals Paracetamol and Aspirin not only caused morphological but as well as physiological effects on Hydra. Hydra showed slowing down of activity as well as inhibition of feeding mechanisms. Although in very minute quantities, the pharmaceuticals do in a way affect the invertebrates adversely and may eventually lead up to bio magnification in upper trophic levels as invertebrates are a major part of the food cycle.

