1. INTRODUCTION

Nepal, situated along the southern slopes of the Himalayan mountain range, is a fascinating landlocked country. It is situated between India to the south, east and west and the Tibetan region of China to the north. It is located between longitudinal 80° 40' and 88° 12' E and between latitude 26° 22' and 30° 27' N. It is roughly rectangular in shape and occupies a total area of 147,181 sq. km.

From the recent taxonomist revision, Taft (1955) prepared a checklist of the fishes known from Nepal. His list contained 95 species, representing 13 families. Further De Witt (1960) elaborated the Taft's checklist by adding some new species. His list included 102 species representing 21 families. Majpuria and Shrestha (1968) published a paper on fresh water fishes and fisheries in Nepal. According to Shrestha (2008), there are 232 fish species in Nepal belonging to 98 genera, 35 families and 11 orders.

Fishes are well defined group of vertebrates. They inhabit all kinds of environment. They have a very special place in human history and civilization. Fishes are the primitive group of the cold blooded vertebrates with gills and fins. There are at least 27,800 species of fish in the world of which about 10,000 are fresh water fish species. These figures probably underestimate the true number, as more species continue to be described. Thus, there may be over 35,000 species of fish world wide (Shrestha, 2008).

Nepal has rich freshwater sources, which constitute snow-fed rivers, lakes and torrential hill-streams and slow moving rivers. Rivers in Nepal covers an area about 395,000 hectares. A number of relatively small to medium sized lakes occur in various parts of Nepal covering an area of 5,000 hectares and numbers of relatively small reservoirs have been constructed, which covers an area of about 1,500 hectares. There are 51,300 hectares of marginal swamps and paddy fields (DoFD, 2001/2002). It is estimated that there are altogether 6,000 rivers. The Koshi, karnali, Narayani and Mahakali are the main river systems. Other important rivers are Bagmati, Kamala, Babai, Manohari, Rapti, etc.

The aquatic ecosystems of Nepal offer excellent habitats to at least 186 indigenous and 11 exotic fish species of high economic, environmental and academic value (Shrestha 1995; Subba and Ghosh, 1996; Shrestha, 1999).

For fishing purpose different types of fishing gears/implements are used in Nepal. Fishing in the waters of Nepal, one and same kind of fishing gears may not be applicable to fish all round the year. A large number of fishing implements have been devised to suit the water resources. Important fishing implements include cast net (Jal), gill net, bamboo fish trap (Dhadia), rod and line, lure (Passo), scooping net (Ghorleng), lift net (Thakauli) etc.

Nets: A net is a piece of webbing, in which the twins are intersected into rectangular meshes, gives a certain form. Fine nylon threads and small metallic weights of iron or leads are used to prepare a net. Cast net, gill net and drift net are the examples of nets used in Nepal. This method is effective in ponds, streams and river.

Diversion of River Channel: In low water phase fishermen divert small side branches of the river from one place to other, thus creating practically fishing channel to scoop fish.

Lure (Paso): During low water phases (January to April), a special kind of baited nylon loop is used to trap fish. Live baits such as fish, shrimp, earthworms, stonefly and mayfly larva are also used in looping. Fish entering the baited loop get stuck by its dorsal and pectoral fins. Fishes are looped in morning and evening.

Bow and arrow (Ban hanne): This method is applied is applied where the water level is low. It is made up of by thin iron rod, making it a shape of arrow. It functions like arrow. This method is used to catch *Gagata, Channa* etc.

Fishing Rod (Balchi Hanne): It consists of three parts: Hook, line and rod. Hook (Balchi) is prepared generally by the fisherman from the rims of umbrella, but now a days it is available readymade in the market. Fine nylon thread is tied at curved tip of the rod and a hook at the distal end of thread. Different types of bait are fixed to the hooks. It is very useful in deep and muddy waters. However, it is very time consuming. This method has

great advantage in finding the predator fishes and scavengers. A single man may operate several rods at the same time.

Electro Fishing: In this method, electricity is applied to catch fishes from shallow rivers and streams. In electro fishing an electric field is produced in water between two electrodes: anode and cathode. The fishes between these electrodes get affected by the electric field which is later collected. The person may stun himself if electro-fishing is improperly carried out. Fish can be sampled with electrical fishing gear from dark underground caves and deep crevices and weedy marshes, where netting is impossible. It is not possible to make electro fishing in deeper water more than 3 meter.

Use of explosive: A number of small and large road construction projects are underway in Nepal. Such projects are provided with explosives to facilitate their construction work, but unfortunately the explosives are often misused in killing fish in large numbers. They are killed together with other aquatic animals, and this is accompanied by damage to the habitat, which recovers only slowly.

Water Poisoning: Poisons are commonly used for fishing in different water bodies as the application of poison is the easy method of fishing. Use of the fish poison is very old practice in the history of mankind. Poisoning should be done with caution to avoid harmful effects to human beings, livestock and the surrounding environment (Shrestha, 1997).

Fishes are killed by poisoning with chemicals viz. aldrin, dieldrin, endrin, BHC, DDT, phosphamidon, thiodine, malathion, dichlorovinylphosphate(DDVP), etc. Use of fish poison is a non-conventional fishing practice which not only kills the fishes but the whole aquatic fauna. It also causes adverse effect on human health by consumption of fish caught by this method.

Fishing with herbal poison is one of the important poisoning methods of the water body. Many hunters use poisonous plants to stun fish so that they become easy to collect by hand. Some of these poisons paralyze the fish; others are thoughts to work by removing oxygen from the water. The poisons affect the fish on nervous, circulatory and/or

respiratory systems. All over the world, indigenous people use various fish poisons to kill the fishes.

Fish poison is made from plant derivatives. Among them most common is derris plant and the root of this plant is used as fish poison. In this root 5% rotenone is present. Due to this rotenone the respiratory system of fish is hampered and fish die. It also kills all the zooplankton present in the water body but doesn't hamper the phytoplankton which is the natural food of fish. This also helps in eradicating bottom insects like black swimmer, snail, etc. This plant is effective up to 1-5 m depth. This method is widely used even in the swamp, ditches and water logs of the river.

In Nepal different plants are used to kill the fishes in stagnant water. These are Ketuke (*Agave Americana*), Khirro (*Sapium insigne*), Kukur tarul (*Dioscorea deltoidea*), Siundi (*Euphorbia royileana*), Bhimsenpati (*Budhleia asiatica*), Mahua (*Madhuca indica*),Bogate (*Maesa macrophylla*), Surti (*Nicotiana tobacum*), Neem (*Azadirachta indica*), Sirris (*Albizzia lebbek*), Khair (*Acacia catechu*), etc.

Shrestha, (2008) states that the fish poisons are prepared from the stem, bark and fruit of many juicy or latex yielding plants. Timur (*Xanthoxylum alatum*), bark of kaphal (*Myrica esculenta*), stem of Titepati (*Artimesia vulgaris*) and root of Aryli (*Edgeworthia*), Chilly powder (*Caspicum*) are also used as fish poison. Leaves of *Sapium insigne* (khirro), *Agave americana* (Ketuke) are crushed and thrown in to the water. Similarly, bark and roots of *Dalbergia stipulacea* are also used for poisoining fish. The crushed leaves and fruits of *Adhatoda vasica* and *Radia dumentorum* are commonly used in ditches for catching fish. These are the common plants used in Terai region (Shrestha, 1997).The juice of the plant *Budhleia asiatica* (Bhimsenpati) is applied as wash to treat skin diseases. The leaves and flower are used in worship and extract of leaf is used as fish poison (Manandhar, 2002).

Great attempts have been made to understand the general toxicology. Due to alarming increase in the use of fish poison special emphasis is being given to the histopathological evidences of damaged tissue or changes occurring in animals exposed to toxic chemicals. Such studies have also offered opportunity to locate the effects of toxic chemicals on

various organs and organ system of animals, reproductive system being one of them. The present paper records the effect of *Agave americana* on ovary of the fish (*Clarias batrachus*) during pre-spawning phase. Main focus has been given to trace out the histological and structural changes brought by this herb in the ovary of the air breathing teleost *C. batrachus.*

1.1 Description of the herb *A. americana*

Man has been harvesting and utilizing agaves for approximately 9,000 years. *Agave americana* was one of the many species described by Linnaeus in the 1753 edition of *Species plantarum*, with the binomial name that we still use today.

Agave americana is a large and stemless succulent, with leaves that can grow up to 2 m. Leaves are robust and spear-like, and are in a basal rosette. The leaves have sharp hooks or spines on the edges, and very sharp tips. It is grown for many reasons- ornamental, medicinal and agricultural. As originally described by Gentry (1970), family Agavaceae consists of 18 genera and a little over 400 species, many of them native to western North America. One of the most familiar species is *Agave americana*, a native of tropical American. Common names includes century plant, Maguey (in Mexico), or American Aloe (it is not, however, closely related to the genus Aloe). This plant is locally known as 'Ketuke' in Nepal. The name "Century Plant" refers to the long time the plant takes to flower.

Agave americana has several uses: ornamental, medicinal, as a vertebrate poison, agricultural, fodder, erosion control (USDA-ARS, 2010). *A. americana* is grown as an ornamental on all continents, except Antarctica (Nobel 1990). *A. americana* is used in Mexico, Brazil, India and China as a traditional treatment, as it has anti-inflammatory, anti-bacterial and anti-fungal properties and can be used as a diuretic (Parmar *et al.* 1992; Peana *et al.* 1997; Jin *et al.* 2004; Boscolo *et al.* 2010; Rivera *et al.* 2010).

A. americana may have adverse effects on human and animal health (Macdonald *et al.* 2003; Badano & Pugnaire 2004; NPPA 2008; Williams 2008). *A. americana* sap can cause pain and dermatitis in humans if it comes in contact with skin (Kerner *et al.* 1973; Ricks *et al.* 1999). The sap has also been shown to have anti-bacterial, anti-fungal and

anti-inflammatory properties (Parmar *et al.* 1992; Peana *et al.* 1997; Jin *et al.* 2004). The sap is diaphoretic, diuretic and laxative. The plant is used internally in the treatment of indigestion, constipation, jaundice and dysentry.

1.2 General Description of the Specimen

The fresh water fish *Clarias batrachus* (Linnaeus) of the family claridae locally known as Mangur is one of the stronger fish having accessory respiratory organs. Description of the specimen fish: *Clarias batrachus.*
Local name: “Mungri” or “Mangur”

Classification: Leo. S. Berg (1947)

Phylum:	Chordata
Division:	Gnathostomata
Super Class:	Pisces
Class:	Osteichthys
Sub Class:	Actinopterygii
Order:	Osterophyss
Sub Order:	Siluroidea
Family:	Clariidae
Genus:	*Clarias*
Species:	*batrachus*

Clarias batrachus also known as walking cat fish has an elongate body that is broader at the head and tapering towards the tail. *C. batrachus* is omnivorous species that feed on a varied mixture of dried pellets, meaty frozen foods, vegetable matter and almost anything offered. It is highly predatory and will eat any fish it can fit into its large mouth. It inhabits river systems, swamps, pools, paddy field, canals and ditches. It is tolerant of a very wide range of water chemistry and temperature. It can also survive in oxygen-depleted conditions due to its ability to breathe atmospheric air. Walking catfish can be found in a variety of habitats, but they are most commonly encountered in muddy or swampy water of high turbidity (Courtenay et al. 1974, Hensley and Courtenay 1980, Talwar and Jhingran 1991).

According to Khanna (1996), on the basis of shape, size, colour of the ovary at least six maturity stages can be recognized:

Resting phase (Immature): The ovaries are small, thin, thread like, translucent, pale or dirty white in colour. The ovaries occupy only a small part of the body cavity and ova are not visible to the naked eye.

Early maturing phase: Ovaries become slightly larger, thicker, opaque and are light yellowish in colour. They occupy nearly half of the body cavity.

Advance maturing phase: There is a further increase in weight and volume of the ovaries, which have a deep yellow colour. Vascular supply increases and the blood capillaries become conspicuous.

Mature or Pre-spawning phase: The ovaries are further enlarged occupying almost the entire body cavity. They are turgid, deep yellow in colour and a large number of spherical ova are visible to the naked eye through the thin ovarian wall.

Spawning phase: Ovaries are very much enlarged, occupying the entire body cavity. They are turgid and yellowing colour with a large number of translucent eggs. Ovarian wall is very thin, almost transparent.

Spent phase: The ovaries are flaccid, shrinked and sac-like, reduced in volume and have a dull colour. The vascular supply is reduced. Histologically, the ovary shows atretic and discharged follicles.

Usually, females with well developed ovaries were found in July-August. The maximum length of *Clarias batrachus* may be 47.0 cm and the weight 1,190 gm. They are potamodromous and lives in freshwater, brackish water, at the depth of 1 to several meters of range.

1.3 Objectives

Ovary being vital organ its changes under the herbal toxicity becomes very important to study. It may affect the progeny.

General objective

Study the effect of herbal toxicity on the reproductive organ (ovary) of *Clarias batrachus.*

Specific objectives

- To study the histology of ovary of *Clarias batrachus.*
- To understand the physiology of the cell under this herb.
- Histopathological study of ovary of *Clarias batrachus.*

1.4 Study Area

Use of toxic herbs to kill fishes is common in Nepal. Generally toxic herbs are used to collect fishes from shallow waters. *Clarias batrachus* was purchased from fish market, Kalimati, located in Kathmandu valley. *Agave Americana* was procured from the garden of T.U. The research work was carried out in the Central Department of Zoology from June 2011 to September 2011 under the supervision of Dr. Archana Prasad, Central Department of Zoology, Tribhuvan University, Kirtipur, Nepal. Daily observation was kept under notice with the help of record book.

The process of microtomy of ovary for the preparation of permanent slide has been conducted in the Central Veterinary Office (Pashu Chikitsa Nirdesanalaya), Tripureshwor, Kathmandu. The photo of slide was taken in laboratory of CDZ with the help of digital microscope.

2. LITERATURE REVIEW

From the time immemorial, humans explored ways and means to divert the poisons of biological origin to their own advantage. Great attempts have been made to study the general toxicology. Use of the fish poisons is very old in practice in the history of human kind. Ludemann Newmann (1960) reported the acute toxicity of some insecticides to a common carp, *Cyprinus carpio.* Several workers have worked on the effect of organophosphorus pesticides on different organs of *Clarias batrachus*.

The histopathological effects of pesticides on the gonads of certain fishes have been described by Mathur (1972), Saxena and Garg (1978), Pandey and Shukla (1985), Singh and Sahai (1985a; 1986) and Sahai (1989) in all these studies it was the ovaries that were examined.

Duodoroff *et al*., (1953) studied the effects of acute toxicity of some organic insecticides to fishes. Many workers have attempted to study the toxic effects of endosulfan to fresh water fish and found that toxicity varies with temperature. Attempt has beeen made to study the histopathological changes induced by non-lethal exposure of phosphamidon recovery in *Labeo rohita* fingerlings, Medda *et al*., (1992).

Singh and Singh (1990) have reported the effect of thiourea on the serum levels of thyroid hormones during the prespawning and spawning phases of the fresh water, catfish, *Clarias batrachus.*

Heavy metals cause differences in the physiological and chemical properties of fish blood (Hughes *et al*., 1988). The change in fish blood exposed to varying degrees of environmental stressors/pesticides have been recorded in the publication of Mishra and Srivastava (1984), Kumar and Banerjee (1991) and others.

Although, small quantities of zinc are required for normal development and metabolism of organisms, if levels exceed the physiological requirements, zinc can act as a toxicant. Exposure to excess zinc has been reported to bring about biochemical as well as

histological changes in various organs of fishes (Agrawal and Srivastava, 2003; Gupta and Srivastava, 2006).

Singh and Agrawal (1984) reported that the latex of *Euphorbia royleana* has high molluscicidal activity in their paper "Alteration in biogenic amine levels in the snail *Lymnaea acuminate* by the latex of *Euphorbia royleana."* Medicinal properties of some plants including *Euphorbia royleana* and *Madhuca indica* are reported by Baral and Kurmi (2006) in their book “A Compendium of Medicinal plants in Nepal”.

An Indian Journal of Traditional Knowledge "Herbal fish toxicant used by fishermen of Karbi-Anglong district"' Kalita, Dutta and Chaudhary (2005) studied and reported the use of plant, *Polygonum hydropiper* (Smartweed) as fish toxicant for catching fish from natural aquatic resources as well as for removal of uneconomical fishes from the aquaculture pond.

Karki and Rai (1982), studied the poisoning constituents of *Sapium insigne* and other plants used as fish poison, in the paper “Observations on the effectiveness of some local plants used as fish poison”. Ambedkar and Munia (2009) studied the piscicidal activitty of methanolic extract of *Capparis stylosa* on the freshwater fish *Channa punctatus*.

Daniel and William (2010), provides coverage of toxic effects in the central nervous, immune, neurobehavioral and reproductive systems as well as describing general mechanisms of toxicity in " Target Organ Toxicity in Marine and Freshwater Teleosts Systems".

A. americana sap can cause pain and dermatitis in humans if it comes in contact with skin (Kerner *et al.* 1973; Ricks *et al.* 1999). The sap has also been shown to have anti-bacterial, anti-fungal and anti-inflammatory properties (Jin *et al.* 2004; Parmar *et al.* 1992; Peana *et al.* 1997). A multitude of plant species are known to possess chemicals toxic to fish, and evidence suggests that certain plant species have different effects depending on which variety of fish are targeted (Van Andel, 2000).

Dimmit (2000) states the beneficial uses and toxicological property of family Agavaceae in the article “A Natural History of the Sonoran Desert”. He explains the edible and medicinal uses of the *Agave.* The juice of the more virulent agaves has been used as fish poison and arrow poison. Similarly, “Agave Plant Health Benefit” a research paper by Sahelian (2008) explains the phytochemical analysis and anti-allergic study of *Agave.*

Mehta, (2001) has studied the ovarian morphology and length-weight relationship of *Xenentodon cancila* (Ham). Pesticidal activity and phytochemical analysis of effective herbs of Nepal has been observed by Pandey, (2010) in his dissertation. He dealt with fish poisonous herbs, "Ketuke" being one of them, their toxic strength and their wild use as fish poison. Similarly, Mukhia (2010) and Bhattarai (2010) studied the impacts of raw and processed water on different organs of the fish, *C. batrachus* and analysed histopathological and histochemical changes brought to the ovary, stomach and intestine of fish.

According to Khanna (1996), in *Clarias batrachus*, the ovaries are paired elongated sac-like structure lying in the body cavity, ventral to the swim bladder. They are flattened ovoid bodies, each measuring about 4-6 cm in length, about 2 cm in width and 1cm in thickness. They are attached to the body walls by the means of the mesovarium. The anterior end is free but their caudal ends may become united into one. The hinder end of each ovary is continued posterior into a short oviduct. The oviducts fuse and open to the exterior by a separate genital aperture or by a common urinogenital opening.

Generally, both the ovaries are of equal size, but occasionally they are unequal also. They are thin, flaccid and translucent when immature, but on maturity, they become enlarged and lobulated, while the ripe ova are seen bulging out. The juice from many species of *Agave* can cause acute contact dermatitis. In human, it may produce reddening and blistering lasting 1 to 2 weeks. Episodes of itching may recur up to a year, thereafter, even though there is no longer a visible rash

With the respect of advancing time the smooth of the ovary gets disrupted and ripe ova bulges out adhering the granular appearance visible through the transparent ovarian wall. The color changes in different phases of its annual reproductive cycle like, it was

yellowish in early stage of development but it changed to reddish brown as the degree of vascularization increased in the latter stage of development.

Histologically, the ovary is made up of an outer wall with developing oocytes and encloses a cavity. The outermost layer consisting of connective tissue is peritoneum, which is so thin that it is difficult to different from adjoining layer. The middle layer is called tunica albuginea which is made up of connective tissue, muscle fibres and blood capillaries. During the pre spawning season, tunica albuginea becomes thickened and same gets thin during the spawning time. The germinal epithelium, which is the innermost layer, consists of a single layer of cuboidal cells which possess scanty cytoplasm and a large nucleus with nucleolus.

The development of a new crop of oogonia takes place in ovigerous lamella which is the part of germinal epithelium. The earlier stage of the oocytes is mainly found in the proximity of the ovarian wall while advance ones lay near the lumen cavity.

The wall of the ovary is fairly thick during the non-breeding season but become highly vascular during the spawning period. The germ cells or oogonia are found in clusters. As the oogonia increase in size, there is an increase in the quality of ooplasm. The developing eggs are known as oocyte. Considering the nuclear and cytoplasmic changes he distinguished oocytes into different growing stages before they attain their maturity. These are the followings:

Stage 1 (S I):
These oocytes are more or less spherical in shape and frequently found to be in clusters along the margin of ovigerous lamella. They are provided with a large distinct nucleus containing nucleoli and numerous chromatin threads. These oocytes are found to be in variable numbers throughout the years.

Stage 2 (S II):
There is the further increase in the size of the oocytes, number of nucleoli and basophilia of the cytoplasm. Several small nucleoli of various sizes are seen along the periphery of the nuclear membrane. Many oocytes at this stage, process a yolk nucleus, lying near to

the nuclear membrane in the cytoplasm. Later, the yolk nucleus moves towards the periphery of the oocyte.

Stage 3 (S III):

This is still large in size, and is distinguished by the appearance of the thin layer of follicular cells around the cytoplasm. A few nucleoli pass out of the nuclear membrane, and are seen in cytoplasm of the oocyte.

Stage 4 (S IV):

There is further increase in the size of the oocyte and a large number of small, clear vacuoles called the yolk vesicles appear in periphery of the ooplasm. The vesicles appear empty in the early stage and are not stainable. Many oocytes show an undulated nuclear membrane, and pass out to the ooplasm.

Stage 5 (S V):

As the oocyte grow further, the yolk vesicles increase in number and fill the entire ooplasm. A vitelline membrane of zona radiata is also clearly visible, between the ooplasm and follicular layer of zona granulosa. Nucleolar extrusion continues at this stage.

Stage 6 (S VI):

This is characterized by the appearance in the form of minute granules in the extra vesicular ooplasm. They appear first in the peripheral region and accumulate there in large numbers. Then yolk proceeds centripetally, till the whole ooplasm becomes impregnated with them. The yolk granules fuse to form large globules, and the oocyte is of considerable size. A thin layer of fibroblast, known as theca is also distinguishable outside the follicular layer.

Stage 7 (S VII):

There is a heavy deposition of yolk globules which are fairly large in size. The yolk vesicles also fuse to become large. The nucleus migrates gradually towards the periphery. Some yolk vesicles push towards the periphery of the egg and form cortical alveoli..

However, these oocytes are of various shapes depending upon the stresses imposed on them and are found in a large numbers throughout the year. The nucleus contains about 2-8 nucleoli together with some fragmented chromatin materials. The cytoplasm is widespread and stains immensely with eosin while the nuclear materials with haematoxylin.

3. MATERIALS AND METHODS

For the study of toxic strength of the *Agave americana*, the sample was collected locally i.e. from T.U garden. The specimen was identified with the help of local people as well as Botanist of taxonomy. The collected sample was cut into pieces and dried in aerated shade and stored at room temperature. The dried sample was powdered by grinder in the laboratory of Central Department of Chemistry, T.U, Kirtipur. The fine powder was kept safe for experimental use.

Adult fish *Clarias batrachus* weighing 250±5 gm and 30 cm in length were purchased from the fish market located in Kalimati of Kathmandu valley in the month of July, 2011 and were acclimatized in a clean aquarium containing tap water in the laboratory of CDZ for fifteen days.

To observe the effect of *A. americana* on the ovary of *Clarias batrachus*, the fishes were kept on three different aquarium, each aquarium filled with 30 liters water. To determine lethal and sub-lethal concentration, the powder of *A. americana* was provided to fishes of aquarium in different quantities. The fishes of aquarium were treated with 3 gm, 4.5gm and 6gm ketuke extract separately. Lc50 was calculated at 4.5 gm, so 3gm of ketuke extract in 30 litres of water was considered as sub-lethal concentration. All the fish were fed with protein diet i.e. liver of chicken and herbal extract weighing 3 gm was mixed well with the help of glass rod in the aquarium. The water of the aquarium was changed every day in the morning after 24 hours before feeding. It was carried out daily by netting fishes on to the other aquarium provided with the same environment. The aquarium was kept clean by changing the water every 24 hours. The experiment was conducted for 19 days. The fish were sacrificed after treating them for 24 hours, 48 hours, 72 hours and 96 hours. Before sacrifying the fishes, they were kept unfed for 24 hours.

The sample tissue i.e. ovary was collected from each hour treated fish and preserved in fixative to undergo the further observation. Meanwhile, the behavioural pattern of the remaining fishes were also observed and written in a record book to get the best output of the study.

3.1 Methods for Histological Study

3.1.1 *Fixation and microtomy*

Before undergoing fixation and microtomy, objects to be studied under microscope needs certain preparation like fixative, block preparation, sectioning, staining and mounting etc. Fixative: A fixative is a chemical which kills tissue with a minimum changes in volume, structure and chemical makeup. It hardens the tissue with post mortem changes in shape, size and also renders some substances insoluble.

Bouin's fluid (alcoholic): The fixative was prepared by taking, following chemicals together in a clean dry tube.

(Saturated) Picric acid (1.4 % aq.)	75ml
Commercial formalin	25ml
Glacial Acetic acid	05ml

After 24 hours of fixation in the above fixative, the tissue i.e. ovary was washed under running water for next 24 hours. Then, tissue were dehydrated in 50% alcohol (3 changes at one hour interval each) followed by 70% alcohol. Thereafter, tissues were further dehydrated (90% and absolute alcohol) and cleared in Xylene and finally embedded in paraffin wax (M.P. 58-60°C). The block ready for sectioning was finally adjusted on the microtome machine and the tissues were sectioned at the thickness of 7 micron.

Staining: Sections of tissue were stained with two dyes to bring contrast between different histological structures. This makes detailed study easier. Staining with two dyes is known as double staining. The two dyes used for double staining were haematoxylin and eosin.

3.1.2 *Staining Procedure*

Xylene (5min) → 100% alcohol (2min) → 90% alcohol(2min) → 70% alcohol(2min) → 50% alcohol(2min) → Distilled water → Delafield Haematoxylin → tap water → Acid water(differentation) → Distilled water → 50% alcohol(2min) → 70% alcohol (2min) → 90% alcohol(2min) → 100% alcohol (2min) → propanol → Xylene (5min).

3.1.3 *Permanent Slide Preparation*

The slide with the paraffin ribbon containing sections of tissue was dipped in xylene for about 5 minutes. The paraffin dissolves. The slide was transferred to absolute alcohol.

Haematoxylin is in aqueous medium. Presuming eosin 70% alcohol the procedure to be followed is given below.

The tissue was dipped in each grade of alcohol for about two minutes with two changes in the absolute alcohol. Time required for staining and differentiation was determined by trial. A small amount of DPX was put on the slide, depending upon the size of the cover slip to be used. The amount should be just enough to form a very thin film on the slide. The cover slip was put on it. The air bubbles locked between the cover slip and slide was removed by leaving the slide overnight on the hot plate.

4. RESULTS

To achieve the objective or purpose of the study, the fishes that are to be examined were kept in aquarium for acclimatization. For the first day, the behaviour pattern of the fish was highly aggressive with jerky movement and tried to escape from the aquarium. This may be due to the environmental fluctuation, but later they all came to normal as they adjusted themselves to that environment.

As we know, the specimen fish was highly carnivorous and detritus feeder in nature, fish fed voraciously on the provided meal to them.

The behaviour pattern of fish and histological changes in the ovary of fish kept under various conditions are traced below:

4.1 Ovary under controlled condition

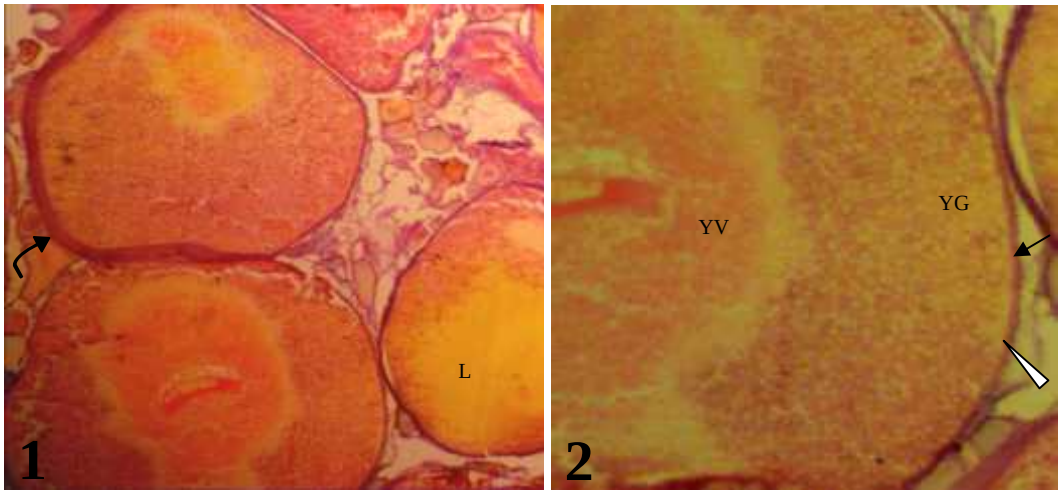

Fig. 1 and 2: Section of ovary showing normal oocytes
comprising theca (↱), zona granulosa (↙), zona radiata (◥)

The fish kept under controlled condition exhibited normal behaviour. It exhibited normal movement and feeding.

The histology of the ovary of the fish revealed no significant change. All the oocytes were in normal condition. Different stages of the oocytes were seen from the germinal epithelium towards the centre of the ovary. Zona granulosa and zona radiata was well organized. Theca appeared fibrous thinner and irregular. Yolk globules (YG) and yolk vesicles (YV) were clear and well organized. There was no appearance of cloudy mass over the oocyte, no vacuolization neither clumping of the oocyte.

4.2 Ovary of *C. batrachus* treated with *A. americana* for 24 hours

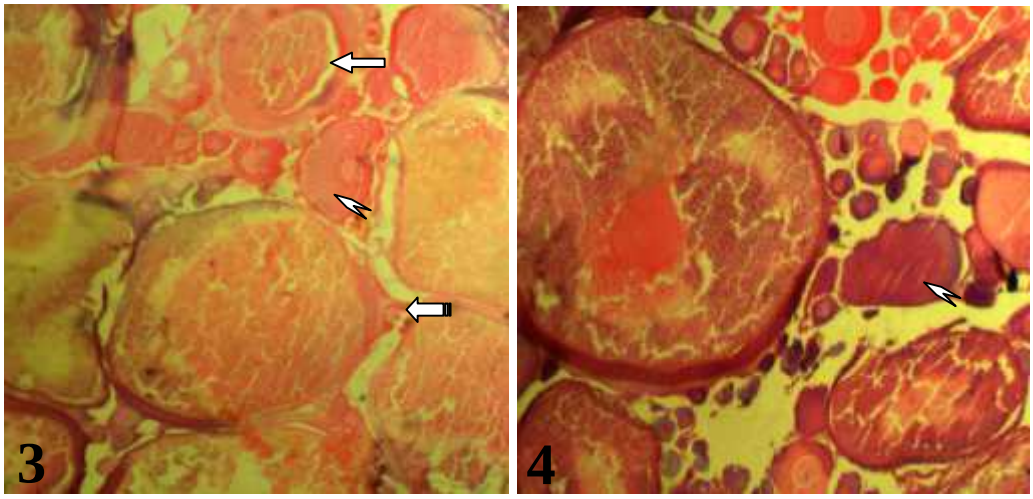

Fig. 3 and 4: Section of ovary showing atretic oocytes (⇘), cytoplasmic shrinkage (⇦) and bulging of follicular epithelium (⇦)

When the fish was treated with powder of *A. americana* for the first time, they showed abnormal behaviour such as excitement, hyper-activities and hypersensitivity. This may be due to change in the environment. They were restless and exhibited violent swimming activities and tried many times to jump out of the aquarium. They often came to the water surface and exhibited gulping activity. The slimy secretion on the surface of the body occurred with reduced feeding from the normal which may be due to toxic effect of herb.

Histological study of the ovary of *C. batrachus* after treatment with *A. americana* for 24 hours showed atretic follicles. A portion of atretic follicle with hypertophic condition of zona granulosa and zona radiata was seen. The ooplasmic shrinkage was not very prominent but many prominent channels and vesicles were formed in the cytoplasm. Bulging on the surface of the oocyte was seen.

4.3 Ovary treated for 48 hours

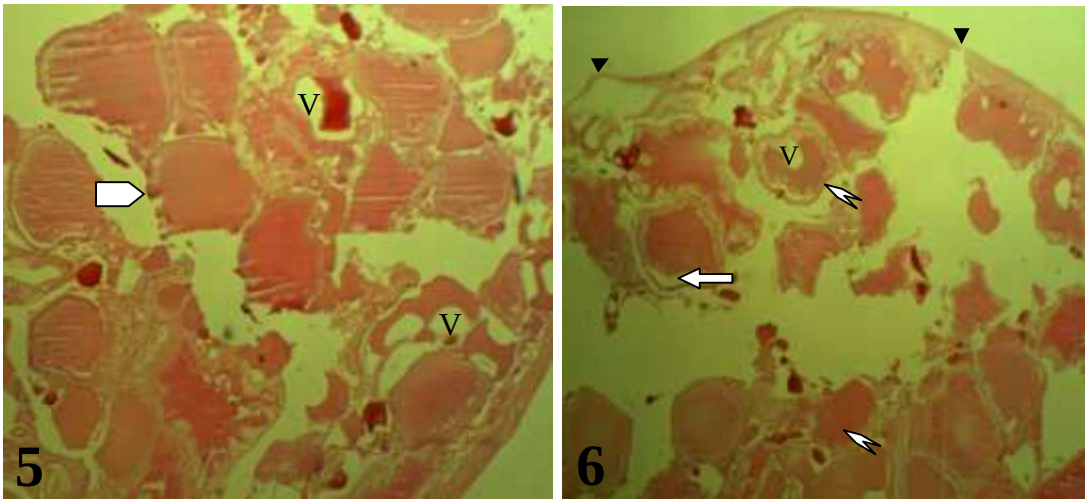

Fig. 5 and 6: Section of ovary showing atretic oocytes ( ) with vacuoles (V), cytoplasmic shrinkage ( ), dissolution and necrosis ( ) of follicular epithelium, etc.

Fish were restless and showed erratic movement. They seem to be tired and weak. They stopped feeding. Excessive mucus secretion on the body surface could be seen. They exhibited increased gulping activity.

Histological studies of ovary revealed large number of atretic follicles. The atresia of the oocytes is characterized by the appearance of irregular vacuoles, liquefaction of the cytoplasmic contents and disappearance of nucleoli. The cytoplasmic shrinkage was very prominent making gaps between ooplasm and cell boundary. The cell boundary seem as they were merging into one another. Appearance of cloudy mass over oocyte could be seen. Necrosis of oocyte was seen to occur. Germinal epithelium seems to be discontinuous and bulging.

4.4 Ovary treated for 72 hours

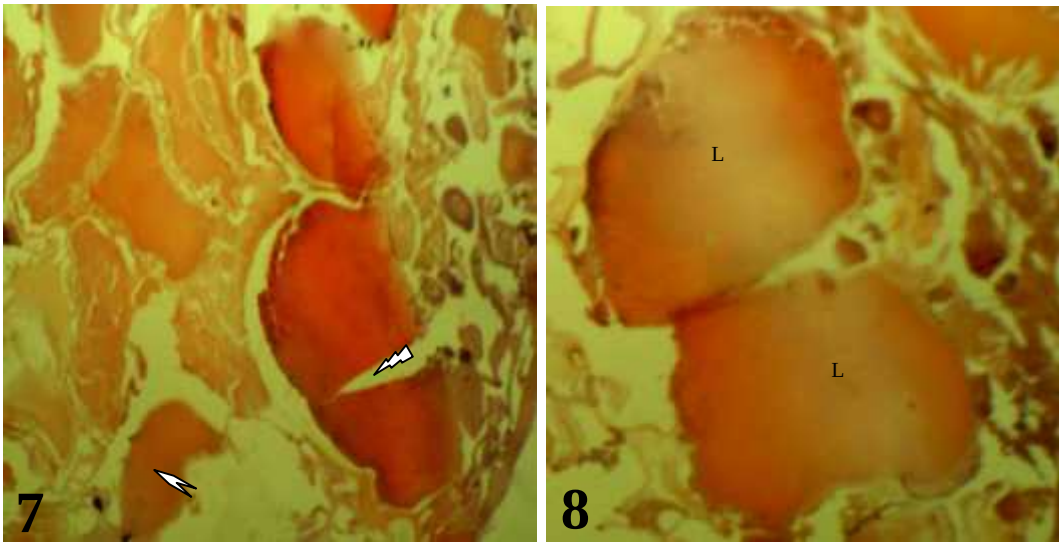

Fig. 7 and 8: Section of ovary showing atretic oocyte (↯), cloudy appearance and lipid (L) on the surface of oocytes

Fish was sluggish, lethargic, stopped feeding and excessive secretion of mucus appeared. The fish exhibited vertical positioning with head above the water surface and loss of equilibrium.

Follicular atresia was maximum. The oocytes of all the growing stages were severely damaged. Dissolution of the follicular cells was most common. Cloudy mass over the follicles appeared. Follicular cells could only be seen in outer margin. Bifurcating of oocyte was seen. Lipid level seems to be less as the staining was light.

Fish died between 72 hours and 96 hours which might be due to some disturbance in the endocrine /hormonal imbalance.

5. DISCUSSION

Histological section of ovary of *C. batrachus* kept under controlled condition showed normal ovarian tissue and normal oocyte distribution. The histological study of the ovary of fish, *C.batrachus* exposed to sub-lethal concentration of *A. americana* for 24, 48, 72 and 96 hours revealed several structural abnormalities or alterations like cytoplasmic shrinkage, vacoulization and degeneration, necrosis and rupture of follicular epithelium. The same result was also found by Dutta *et al*. (1994), Guraya (1993) after exposure to the sub-lethal concentration of malathion for 24 hours who reported follicular atresia, clumping of cytoplasm, cytoplasmic shrinkage and fusion of the oocytes after the treatment of malathion in *Heteropneustes fossilis*. They also observed reduction in estrogen level. In the present study, these changes are most common.

Ram and Sathyanesan (1984) expressed that mercury was reported to reduce gonadotrophin release, which cause impairment in yolk formation by the oocytes of *Chana punctatus.*

According to Khanna (1996), the main function of the follicular atresia is to limit the number of oocyte that would undergo vitellogenesis and become mature for ovulation. The same case was observed in all treated ovaries (Fig. 3, 4 6 & 7). Atretic pre-vitellogenesis follicles, possibly give origin to the interstitial cells of the ovary, and may secrete steroid hormones required for the growth and maturation of the normal follicles. Histochemical and in-vitro studies have failed to demonstrate any enzyme activity in these atretic follicles. Further studies may finally decide whether the atretic follicles of the teleost are associated with degeneration and absorption of the yolk only or the have some other function also.

Pathwardhan and Gaikwad (1990) observed various atretic properties of the ovary of mosquito-fish. The results indicated that sub-lethal exposure of malathion may not kill fish, but causes severe ovarian damage, reducing the number of viable eggs and reproductive potential.

Baronia *et al*. (1993) observed enlarged germinal epithelium, vacuolization and irregular and damaged ovum and large cystic follicles on the ovary of albino rats while studying long term malathion administration. However, in our case the evidence i.e. vacuole formation was seen in ovary of fish treated for 48 hours (fig.-5 and fig.-6).

Dhawan and Kaur (1997) reported a decline in the lipid content of the ovary of *Cyprinus carpio* and *Cirrhina mrigala* after exposure to zinc for 60 days. Similarly, Sindhe et al., (2002) concluded that the reduction in total lipid content may be a result of disturbed vitellogenesis, steroidogenis and/or reduced enzyme activity. In present study similar result is obtained i.e. ovary treated for 48 hours revealed decline in lipid level as the staining is light and it is more pronounced in the ovary treated for 72 hours (fig. 8).

In present investigation, the severity of the toxin i.e. ketuke extract in the ovary is found to be time dependent. Initially, less atretic follicles and cytoplasmic shrinkage were observed, the ovaries of fish exposed for 48 hours and 72 hours displayed immense amount of atretic follicles along with cytoplasmic shrinkage, vacoulization and necrosis of oocytes. This result is in agreement with the result of Dutta and Dalal (2008) who reported similar significant changes in the ovary of fish exposed to endosulfan for different period of time with different concentrations.

According to Jegede and Fagbenro (2008) histological sections of the ovary in *Tilapia zillii* fed with the control diet showed normal ovary histology. No pathological lesions were observed and atretic follicles were less visible. Similar to this in present study, the fish under controlled condition showed normal ovarian structure (fig. 1 & 2). In fish fed with neem leaves, there were changes in colour of ovaries, increased atretic follicles, ruptured follicles and necrosis indicating the effectiveness of neem leaves as stirility-inducing agent. In present study, these changes are very common in the ovary exposed to sub-lethal concentration of ketuke for 48 and 72 hours (fig. 5, 6 & 7).

The fish treated with ketuke extract exhibited abnormal behaviour similar as described by Jothivel and Paul (2008). These changes include violent swimming activities, increased gulping activity and excessive secretion of mucus all over the body. The fish often came to the water surface and also tried many times to jump out of the aquaria. None of the

above behaviour was shown by the fish under controlled condition. Jerky, violent movements were noted as a direct result of ionic imbalance caused by endosulfan (Rangaswamy and Naidu, 1989).

Under present research, the atresia of the oocytes is characterized by the appearance of irregular vacuoles, liquefaction of cytoplasmic contents and irregular and discontinuous germinal epithelium. Irregularly thickened granulosa layer, discontinuous zona radiata and fibrous, thinner and irregular theca can be seen in the atretic follicles. Similar result was reported by Mukhia (2010) in his dissertation after treatment of the fish, *Clarias batrachus* in raw water containing excess of ammonia.

6. CONCLUSION AND RECOMMENDATIONS

Thus the herbal poison may have an adverse effect not only on the organ which come in direct contact with the toxin external medium such as scales and gills, but also on the vital organs like ovary, testis, kidney and liver. The study focuses on microscopic changes that occur on oocytes at different stages of development. In this study, the damage done to ovary was minimal at 24 hours. This study showed that the more degenerative changes were seen at 48 hours of experiment. Clumping of cytoplasm appeared after 48 hours of exposure to ketuke. Necrosis and degeneration of follicular cells was observed and follicular cells become loose and ruptured. A large number of atretic oocytes were visible. The ovary of fish treated for 72 hours exhibited more severe condition than 48 hours. All the oocytes were seen to gather towards the outer margin of ovary. Appearance of cloudy mass over the oocytes was seen. Lipid level seems to be very low which may affect the reproductive potential of fish. The death of the fish at 96 hours indicates that it might have its effect on nervous system.

The above findings suggest that the histopathological changes in the ovary might be a reflection of the disturbance in the endocrine/hormone imbalance.

The herbal poison administered to the fishes by the fisherman is very high. In the present study a sub-lethal dose for a short period of time was administered. Still it showed some degenerative changes in the oocytes. The accumulation of the herbal poison was also seen in the tissue. Therefore, I recommend the use of any herbal poison should be well monitored because it passes them to the higher form of animals ultimately to men. Since, its effect was observed on the oocyte degenerative which can be inferred that the amming generations might not be potential.

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