INTRODUCTION

Inland water resources are the most important natural resources of the Nepal Himalaya. Nepal is the second richest country in inland water resources in the world, possessing about 2.27% of the world water resources (CBS 2006). The country has more than 6,000 rivers, which provide a dense network of rivers with steep topographic conditions. All the river systems drain from north to south towards the Ganges. About 5% of the total surface area of the country is covered by water and they are endowed with a great variety of natural fish distribution. Fishes are one of the most important natural resources as food and livelihood for local fishermen community.

Fish are an important resource worldwide, especially as food. Commercial and subsistence fishers hunt fish in wild fisheries or farm them in ponds or in cages in the ocean. They are also caught by recreational fishers, kept as pets, raised by fishkeepers, and exhibited in public aquaria. Fish have a role in culture through the ages, serving as deities, religious symbols, and as the subjects of art, books and movies. In Nepal fishes plays an important role not only from the economic point of view but also from the religious point of view as result of which the market of fisheries are expanding these days. The occurrences of fishes in Nepal have been described originally by Hamilton in“Fishes of the Ganges” in the year 1822. Many people have worked on the fishes of Nepal in the due course of time. There are about 27,000 species fishes in the world of which 10,000 are fresh water fish species (Shrestha 2008). Shrestha (1973-78) worked on the fishes of Nepal and reported 114 species from this country. According to the (Shrestha, 2008), there are 232 species in Nepal belonging to 98 genera, 35 families and 11 orders.Nepal has great potential of reverine fishery and rice field fishery.

There are different kinds of fishing gears used in Nepal which are classified as conventional and non-conventional type (Shrestha 1997). Conventional gears include the nets, bamboo traps, rod and line and looping where as non conventional methods includes use of explosive, poisoining and electrofishing. The aquatic life especially naturally distributed fish species are the most threatened biological component mainly due to the use of non-conventional gears like:

Use of explosive:- The misuse of explosives is the main cause for the irreparable damage to the aquatic ecology which not only kills the fish but also other organisms along with the fish of undesirable size.

Electrofishing:- The electric field is created in the water in order to kill the fish. For which two electrodes are dipped in water and a large number of fish can be caught easily however, undesirable aquatic organisms are also killed by it.

Chemical Poisoning:- One of the most commonly used method of killing fish is, the use of poison in the aquatic body. Different types of chemicals can be used such as aldrin, BHC, endrin, DDT, thiometon, phosphamidon dichlorovinyl phosphate (DDVP), thiodine, malathion etc.

Plant poisons:- Besides toxic chemicals, variety of plant poisons have also been used traditionally for this purpose (Karki and Rai 1982). Some of the plants have found to be used by many hunters, gatherer to stun fishes for easy catching by hands. Plants such as Azalea (*Rhododendron species*), Black locust (*Robinian species*), Blue cardinal flower, Castor bean (*Ricinus communis*), Clover (*Trifolium species*), Tobacco *(Nicotiana tabacum*), white snake root *(Eupatorium rugosum*), wild building heart *(Dicentra species*) etc were also used as plant piscicides (Begeley 2000). Shrestha (1997) stated that the fish poisons were 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*) were 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 plants products are first well crushed and grinded near by the aquatic system. The paste is used as fish poison in stagnant and semi stagnant pools, slow flowing streams and rivers for fish catch and is mostly applied during morning and evening hours. Streams would be partly blocked to slow down the water for concentrating the poison without being washed away or diluted by a strong current. The variety of chemical found in these plants will stun fish, when it passes through the gills or in some cases ingested. The fishes come in to the surface and exhibit the abnormal behavior due to may be the nervous breakdown and lack of dissolved oxygen. The fainted fish are collected by fisherman and local people using simple cloth cast net and put in to basket (Pandey 2010). The poison has a nercotic and debilitating action on the fish, and may paralize the fish. At the beginning all the fishes are paralized but those that are in deep pool and those that have not been picked up by fishermen were revived. Plants extracts also alters the physicochemical properties of water bodies which ultimately cause decline of small fishes in streams and rivers. The toxicants released from paste or slurry of these plants not only affects the fishes but also damage the stream biota like periphyton and macrobanthos. A variety of plants having fish poison properties has been known for the countries, especially in South America and Asia. Many have been employed directly to collect wild fish from streams and ponds for the table.

Maguey or *Agave americana* is a native plant from Mexico which is called 'Ketuke' in Nepali, but is now cultivated in many parts of the world. This plant, which is also known with the name of century plant or American aloe, is neither an aloe nor a cactus, as it is sometimes erroneously believed, but pertains to the *Agavaceae* family. Maguey is one of the many species of *Agave* plants that exist in the America. They grow in semi-arid environments from the sea level to an altitude of about 9000 feet (Karki and Rai, 1982). *Agave americana* is a large, rhizomatous succulent that grows in a wide range of conditions including cliffs, urban areas, woodlands, grasslands, riparian zones, beaches and sandy areas, and rocky slopes. *Agave americana* is tolerant of wind, salt, high temperatures, and extreme drought. It can grow in shallow, very dry, low fertility soil and can colonise bare sand. It is grown for many reasons- ornamental, medicinal and agricultural. In South Australia *Agave americana* mainly invades disturbed sites, road sides and coastal vegetation. Archaeological evidence indicates that agave was used as early as 12,000 years ago by archaic foragers groups, to obtain fibres for clothing, bags and to make tools. There is no direct evidence of domestication of agave, but it seems that only a handful of species, of the hundreds existing in nature, have been fully domesticated. The use of this plant for fish killing is an ancient practice however very little is known about it. Agave can cause severe dermatitis (Shrestha 1997). The juice of more virulent Agaves has been used as fish poison and arrow poison (Dimmit 2002). Local people use its fleshy leaves in the body of water in order to paralyze the fish only after grinding. After the use of it, fish are seems to be unconcious and come to the surface making easy for collecting and thus is a very common among the different tribes.

The present study was focused on the effects of Maguey or *Agave americana* or ‘Ketuke’ on the kidney of fish. Kidney is an important organ of any vertebrate which work for the elimination of the nitrogenous wastes from the body and also helps in the regulation of body fluid and electrolyte balance of animals (Khanna 1996). This organ of fish consists of renal capsule, neck segment, proximal segment, intermediate segment, distal segment and collecting tubule as main parts. Any abnormality in the kidney produces overall change in the body function. Thus histopathologial study is carried out in medical and veterinary science as a diagnostic tool. In the present study, histopathological analysis was done out in order to find out the effect of herbal pesticide in the kidney of *Clarias batrachus.*

1.1 LIMITATION OF THE STUDY

The lab work for the experiments was done on the laboratory of Central Department of Zoology Kirtipur. Histological study had been carried out in central veterinary office, Tripureshwor, Kathmandu. The present study deals with the analysis of important changes on the histology of kidney due to the effect of herbal toxicity (*Agave americana*). The effect of ‘Ketuke’ on the glandular structure and secretion of the kidney of fishes of different species could not be done due to the limited time and laboratory access.

1.2 AIMS AND OBJECTIVES OF THE STUDY

1. GENERAL OBJECTIVES
    - To find the effects of *Agave americana* on the kidney of *Clarias batrachus.*

2. SPECIFIC OBJECTIVES
    - Study of the morphology and histology of kidney of *Clarias batrachus*.
    - Histopathological analysis of kidney of *Clarias batrachus*.
    - Study for the chance of replacement of chemical poison herbal pesticides like *Agave americana.*

1.3 STUDY AREA

The study in the effect of *Agave americana* (Ketuke) on the kidney of *Clarias batrachus* was carried out in the Central Department of Zoology (CDZ), Tribhuvan University, which is situated in Kathmandu district of Nepal, under the supervision of Dr. Archana Prasad (Thesis supervisor) from the date July 2011 to September 2011. Daily observation of aquarium was kept under notice with the help of record book.

The Kathmandu valley is situated 85^0 33’ east to 85^0 20’ west and 27^0 42’ north to 27^0 7’ south and is the headquaters of central region (Madhyamanchal).The fishes for the study were purchased from fishery market of kalimati Kathmandu is located in Bagmati zone.

The study of the microanatomy of specific tissue, have been successfully conducted in the Central Veterinary Office (Pashu Chikitsa Nirdesanalaya) Tripureshwor, Kathmandu for the preparation of permanent slide and photo was taken with the help of digital microscope in the laboratory of CDZ.

1.4 DESCRIPTION OF THE SPECIMEN

Clarias batrachus has the following taxonomic position (Leo.s.Berg, 1947)

Phylum: chordata, Division: Gnathostomata, Super Class: Pisces, Class: Osteichthys, Order: Osterophyss, Sub Order: Siluroidae, Family: Claridae, Genus: *Clarias*, Species: *batrachus* Local name- Mungri or Magur

Short description

Members of the family Clariidae are collectively known as "air-breathing" catfishes although they are not the only catfish family capable of breathing atmospheric air. Walking catfish is the most common English name for this species. These are found in a wide variety of habitats including lakes and rivers but are best known for their ability to thrive where many fishes can not. Warm, stagnant, often hypoxic waters such as muddy ponds, canals, ditches, swamps and flooded prairies are common habitat for this fish. Except for occasional forays to the surface for gulps of atmospheric air, this species spends most of its time on or near the substrate.

Magur is typically a uniform shade of grey or grey-brown in colour. The head is flat and broad and the body tapers to the tail. The mouth is broad, although the gape is not great with the fleshy lips. It possesses very small eyes, lengthy dorsal and anal fin that terminate in a lobe near but free from the caudal fin, and pectoral fins with rigid spine-like elements, one each at the fore. It is through the use of these stiff pectoral "spines" accompanied by a back and forth flexion of the body that walking catfish accomplish their ungainly but effective terrestrial locomotion.

An extension of the arborescent organ that supports the fish's gill filaments, this structure facilitates the uptake of atmospheric oxygen by providing support for a number of gill filaments that would otherwise collapse in the absence of the buoyancy of water. This accessory air-breathing organ functions much like a lung. The branching appearance of this organ, reminiscent of a small tree, is the basis for the name "arborescent" organ.

This species is reported to reach a length of 61cm (24 inches) in its native range. It is benthic omnivores, industrious in their search for food and is nocturnal species, which search the bottom with their barbels vigorously shifting through detritus and soft substrates. It consume a wide variety of prey, including eggs or larvae of other fishes, small fishes, and a number of invertebrate taxa such as annelids, crustaceans, and insects. In densely populated drying pools, walking catfish are particularly indiscriminate in their choice of prey items, often seizing and consuming a wider variety of prey than what may normally be available and construct nests made of detritus or submerged vegetation. The male guards the adhesive eggs and free-swimming young. In the native range, spawning is coincident with the onset of the rainy season during which the species may construct nests in the flooded environment.

Agave americana (Ketuke)

Fig.1: *Agave americana* (Ketuke)

Agave americana has the following taxonomic position (Linnaeus, 1753)

Kingdom: Plantae, Division: Magnoliophyta, Class: Liliopsida, Order: Asparagales Family: Agavaceae, Hutchinson Genus: *Agave,* Species: *americana* Local Name-Ketuke

Short description

Agave americana is a plant that belongs to the family Agavaceae with 18 genera and a little over 400 species, and is commonly known as the century plant, maguey, or American aloe (although it is in a different family from the Aloe), is an agave originally from Mexico but cultivated worldwide as an ornamental plant. This plant is locally called

‘Ketuke’ in Nepal.It has since naturalised in many regions and grows wild in Europe, South Africa, India, and Australia.

The misnamed century plant typically lives only 10 to 30 years. It has a spreading rosette (about 4 m/13 ft wide) of grey-green leaves up to 2 m (6.6 ft) long, each with a spiny margin and a heavy spike at the tip that can pierce to the bone. When it flowers, the spike with a cyme of big yellow flowers may reach up to 8 m (26 ft) in height. Its common name likely derives from its semelparous nature of flowering only once at the end of its long life. The plant dies after flowering, but produces suckers or adventitious shoots from the base, which continue its growth.

If the flower stem is cut without flowering, a sweet liquid called *agua miel* (honey water) gathers in the heart of the plant. This may be fermented to produce the drink called pulque. The leaves also yield fibers, known as pita, which are suitable for making rope, matting, and coarse cloth and are used for embroidery of leather in a technique known as piteado. Both pulque and maguey fibre were important to the economy of pre-Columbian Mexico. Agave nectar, also called agave syrup, is marketed as a natural sugar substitute with a low glycemic index that is due to its high fructose content. The sap is quite acidic and can be quite painful if it comes in contact with the skin. It can form small blisters and can cause severe dermatitis (Shrestha 1995) and the juice of it has been used as fish poison and arrow poison (Dimmit 2002).

LITERATURE REVIEW

Scientist were limited to the study of taxonomy, functional anatomy and morphology before the beginning of 16th century , However the study become gradually expanded to the histomorphology, histophysiology, endocrinology, cytology and ecology of fishes after the beginning of 19th century. The credit for the initiation of study of kidney goes to Bowman (1842). Nephrons of fishes are provided with glomeruli condition of some fishes was observed first by Hyrtl (1850). Marshal and Smith (1930) studied the development of glomerule in relation to the habitat.

"New Glandular Structure in the Kidney of *Clarias batrachus* (Linn.)" was studied by Sharma (1968) and mentioned about the gandular structure of the mesonephrons of the air breathing teleost. It explained about the spheroidal multicellular gland inside the renal mass, which is situated adjacent to the extension to the caudal vein and is surrounded by a delicate investing membrane and is constituted by close folding of the epithelial layer so as to form a number of semi parallel ridge of columnar gland-cells separated by narrow interspaces. The chink-like lumen of the gland opens, by a minute orifice into a space surrounding it, and this peri-glandular space discharge, by a funnel-shaped aperture, into a duct situated on one side of the gland.

Attempts have been made to study the histopathological changes. The change in fish blood exposed to varying degrees of environmental stressor/pesticides have been studied by Mishra and Banalata (2008) and Singh *et* al. (2006). Kirubagaran and Joy (1988) had studied about the toxic effect of mercury on the histology of kidney of catfish *Clarias batrachus*. Dimmit (2002) stated the beneficial uses and toxicological property of family Agavaceae in the article "A Natural History of Sonoran Desert". He explained the edible and medicinal uses of Agave.

Manandhar (2002), in the book "Plants and people of Nepal" explained that the juice of plant is applied as a wash to treat skin diseases. The juice of more virulant agaves has been used as fish poison and arrow poison (Dimmit 2002). The book "Fish Catching in the Himalayan Waters of Nepal" and "Ichthyology of Nepal" by Shrestha (1995, 2008) also reports about herbal fish poison.Tamang (2003) mentioned the use of *Agave americana* for Diuretic and anti-syphilitic purpose by Tamang people of Nepal.

Deutsch (1988-2006), documented a concise overview of the contact-poisonous plants in his research work “contact Poisonous Plants of the World”. The first part of the paper briefly introduced the active principles, effects, treatment and geographical distribution. The second part lists about 35 important plant species and described them in details.

Shrestha (1997) stated that, the fish poisons were prepared from stem, bark and fruit of many juicy or latex yielding plants. Bark of Kaphal (*Myrica esculenta*), stem of Titaepati (*Artimesia vulgaris*), Chilly powder (Caspicum) are also used as fish poison.

An Indian journal of Traditional Knowledge “Herbal fish toxicant used by fisher of Karbi-Anglong district, Assam”, Kalita *et* al. (2005) studied and reported the use of plants, *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.

Great attempts have been made to study the general toxicology. Kumar *et* al. (2006) had studied about stress in the haematological parameters in the blood of *Clarias batrachus* (Magur) exposed to two anionic synthetic detergent Rin advanced and geepool.

Histopathological changes in the different tissues of freshwater fish (*Hypopthalmichthys molitrix)* due to Nickle exposure had been studied by Athikesavan *et* al. (2006) and concluded that degeneration of tubular cells, hyperplasia resulted in the tissue of kidney. Similarly Singh and Gupta (2006) have studied the biochemical changes due to the parasitic infection in the liver of fresh water catfish (*Channa gachua*). The pattern of accumulation of cadmium and its affinity to selected tissues - gills, kidneys, liver, skin and muscle - of *Clarias batrachus* exposed to sublethal concentrations (7 ppm) of cadmium chloride was investigated by Jayakumar and Paul (2006).

Histological changes in gills, kidney and liver were used to evaluate the health of the Neotropical fish species *Prochilodus lineatus*, subjected to *in situ* tests for 7 days in a disturbed urban stream and in a reference site, during winter and summer by Camargo and Martinez (2007).

The biochemical profile and pattern of physiological adaptations, suggesting an intermediary status of two fishes (*Heteropneustes fossilis* and *Clarias batrachus*) in the evolution of ureotely in vertebrates, was discussed by Saha and Ratha (2007) in their article entitle "Functional urogenesis and adaptation to ammonia metabolism in indian freshwater catfishes".

Ruhela *et* al. (2008) studied about the histological manifestation in kidney of Clarias batrachus induced by experimental *Procamallanus* infection. Similarly, "Agave plant Health Benefit" a research paper by Sahelian (2008) explained the phytochemical analysis and anti allergic study of *Agave*.

Datta *et* al. (2009) studied the effect of chronic low-level arsenic exposure on the head kidney (HK) of *Clarias batrachus* and determined the changes in head kidney macrophage (HKM) activity in response to arsenic exposure.

Saenphet *et* al. (2009) observed the histopathological changes such as hypertrophy of the epithelial cells of the renal tubules with reduced lumens, aneurisms of the renal tubules, and contractions of the glomeruli in the Bowman's capsule as a result of investigation of effect of acidic mine water on *Anabas testudinus* (Bloch).

Genotoxicity induced by fluoride (F) has been observed in kidney cells of freshwater Asian catfish, *Clarias batrachus* by Tripati *et* al. (2009). Cadmium chloride effect on the histoarchitecture of kidney and liver of *Clarias* was investigated by Ahmad *et* al. (2010) and indicated the different duration dependent histopathological alteration and resulted in the physiologic and metabolic dysregulation.

Pandey (2010) explain the toxic effect of various plants including Ketuke on fish in his dissertation work and concluded that the effect of Ketuke on fish body is due to the presence of chemicals like Alkaloid, tannin, Saponin, Flavonoid, Steroid and Phenols.

Kumar *et* al. (2012) in their article "Alterations in nitrogen metabolism in freshwater fishes, *Channa punctatus* and *Clarias batrachus*, exposed to a commercial-grade λ-cyhalothrin, REEVA-5" mention the comparative mechanisms of ammonia detoxification in both fishes. In fishwise comparison, *C. batrachus* was observed to be more sensitive with respect to the above-mentioned parameters. Another important finding was that unlike the liver, the kidney appeared as one of the primary sites of ureogenesis in fishes.

Similarly Banerjee *et* al. (2012) studied about the Aeromonas hydrophila induced head kidney macrophage apoptosis in *Clarias batrachus* involves the activation of calpain and is caspase-3 mediated.

Fish have been adapted to many different environments and the morphology and functions of many organs, including the kidney, may show extreme variations both macroscopically and histologically (Khanna 1996). Proper knowledge of the anatomy and functions are therefore of the greatest importance as a basis for interpretation of lessions in this organ. This brief introduction of normal structure and function will allow only a few important facts to be mentioned. The development and relative size of the different components may vary considerable, but as in higher vertebrates, the following components may be identified: I) glomerulus, 2) tubules (neck segment, proximal and distal tubules) and 3) collecting ducts.

The relative length and differentiation between the different components may differ considerably between species and changes may also occur during the normal life cycle. For example, aglomerular fish do exist in the marine environment and the number and size of glomeruli may vary considerably in fish that migrate between the marine and freshwater environment. Collecting ducts are differentiated from the tubules by their cuboidal epithelium, larger diameter and the presence of some smooth muscle fibres and connective tissue surrounding them.

Blood supply:- The kidney is supplied with both arterial and venous blood. The capillaries in the glomerulus are branches of the renal artery. Venous blood from the kidney drains to the caudal cardinal vein (Kotpal 2006).

Haematopoietic and Iymphoid tissue:- This tissue dominates in the anterior portion of the kidney while the number of nephrons increases caudally. In the rear 2/3 (approx.) of the kidney, the nephrons are interspersed between the Haematopoietic interstitial tissues. This is a highly active tissue where mitoses frequently are observed, in particular in young fish. Varying numbers of melanomacrophages may be found in this tissue, in many fish they are organized as melanomacrophage centers. Typically, their number may be high in older fish and their number may also increase considerably after disease. In this tissue are also eosinophilic granular cells (EGC). Their number may fluctuate considerably and sometimes they may be the dominating cell type (Srivastava, 1985).

The Glomerulus:- The components of the complex glomerulus are the fenestrated capillary endothelium, the glomerular basement membrane, the podocytes (visceral epithelial cells) and the parietal epithelial cells that line Bowman's space where the plasma filtrate first collects. The entire glomerular tuft is supported by the mesangial cells of mesenchymal origin that are biologically very active and may proliferate, contract and secrete biologically active mediators. The fenestrated epithelium and the podocytcs form a filtration slit where the ultrafiltrate are made.

Endocrine tissue. The following endocrine organs are usually found in the kidneys:

The corpuscles are usually located near the kidneys and may be fused into the organ. Their number and size may vary considerably. They are separated from the kidney tissue by fibrous tissue and from the surrounding capsule. Strands of fibrous tissue extend into the corpuscles dividing them into smaller lobuli. The functional unit consists of large, polygonal parenchymal cells with a large, central nucleus.

The location of the interrenal cells may vary considerably and may be spread over most of the kidney. They usually stain deeply eosinophilic and have a round nucleus with a prominent nucleolus. Lipid inclusions that may hypertrophy during sexual maturation are common in some species.

The suprarenal cells in histological preparations are typically rather large, pale staining cells with a granular cytoplasm. They are often located close to the interrenal cell.

MATERIALS AND METHODS

The fishes required for the experiment were purchased from the fish market located in Kalimati of Kathmandu valley. Adult fifteen fishes of weight 250+\- 5gm and the length of 30 cm were acclimatized for fifteen days in the clean aquarium containing tap water in the laboratory of CDZ in the month of July 2011.

The present study is carried out by taking *Clarias batrachus*, an experimental fish as its care level is very easy. It has ability to tolerate a wide range of water parameters including pH, temperature and hardness and can tolerate higher nitrate levels than other fish and is hardy enough to withstand the cycling process which will readily eat prepared foods and has no special care requirements. Thus most of the laboratory experiments are conducted with it.

The leaves of (Ketuke) *Agave americana* were collected from Tribhuvan University premises and the specimen were identified with the help of local people as well as expert Plant Taxonomist. The powder of ‘Ketuke’ was obtained by grinding the leaves after cutting it in to the smaller pieces and were dried in aerated shade. The process of grinding was carried out in the grinding machine of Central Department of Chemistry (CDC), TU, Kirtipur. The fine powder was then kept safely for the experimental use.

To observe the effect of ‘Ketuke’ on the kidney of fish, the fishes were kept in aquarium with the powder prepared for different period of time i.e 24 hours, 48 hours, 72 hours and 96 hours at different concentration. The three sets of fishes were kept, one set was treated with 0.1gm/l, second 0.15gm/l and last 0.2gm/l of extract. Lc50 was calculated at 0.15gm/l. In present study, 3.0gm, 4.5gm and 6.0gm of ketuke extract were kept in 30l of water to prepare 0.1gm/l, second 0.15gm/l and last 0.2gm/l as sublethal dose. All the fishes were fed with protein diet, liver of chicken at the rate of 50 gm per fish per day. The water was changed everyday in the morning time before feeding. The experiment was conducted for 19 days and before the sacrifising of the fishes, they were kept unfed for 24 hours.

The tissue of kidney was collected from each provided condition and preserved in Bouin's fixative. The tissues were dehydrated and preceded for the microtome study. Sections were made 5 micron thickness. Meanwhile, the change behavioural patterns of the remaining fishes were also closely observed and were recorded in note book so as to meet the objective of the study.

METHODS FOR THE HISTOLOGICAL STUDY:

Fixation and Microtomy

First of all the tissue of kidney to be observed under the microscope required preparation of fixative block, sectioning, staining and mounting etc. and then only the process of fixation and microtomy was carried out.

After sacrificing the animal, kidney was taken out for the fixation in Bouin's fixative. The size of kidney was about 2cm in length and 250 mg in weight.The fixative used in tissue for fixation is found to be very useful because it kills tissue with minimum change in volume, structure and chemical make up as well as hardens the tissue with post mortem change in shape and size and also renders some substances insoluble.

The Bouin's fixative (alcoholic) was prepared by mixing up the three chemicals in definite proportion as: (Saturated) Picric Acid (1.4% aq.) -75ml, Commercial Formalin-25ml and Glacial Acetic Acid -5ml.

The tissues were washed in running water for next 24 hours after fixing in Bouin's fixative for 24 hours. Then the tissue were dehydrated in 50% alcohol by three changes with the interval of one hour each followed by 70% alcohol. Then after again the tissue were subjected to the dehydration with the 90% and absolute alcohol and then the tissue was cleared in xylene and finally embeded in parafin wax (melting point 58-60^{0}C).The tissue were adjusted to microtome machine and sectioned at the thickness of 5 microns when the block was ready. The microtome of the kidney tissue was done in the Central Veterinary Office (Pashu Chikitsa Nirdesanalaya) located in Tripureshwor, Kathmandu.

Staining

The Double staining process was done for making the detailed study easier. The two dyes used for double staining technique was haematoxylin and eosin which produces different colour in the different parts of tissue such as haematoxylin makes the nucleus blue in contrast eosin make the cytoplasm pink with orange\red red blood cells (RBC) and pink colour of collagen fibres.

Staining procedure:

Permanent slide preparation:

To obtain the permanent slide of the tissue the slide with parafin wax containing section was dipped in Xylene for 5 minutes, as the paraffin dissolved, the slide was transferred to absolute alcohol.

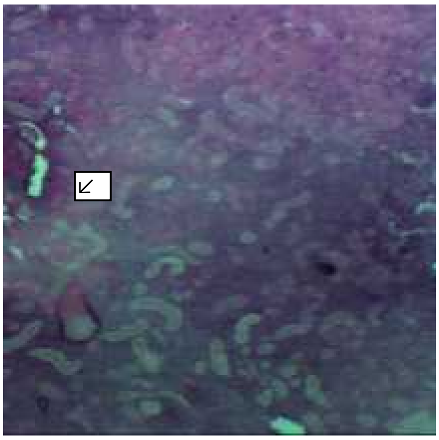
Haematoxylin is in aqueous medium. Presuming eosin in70% alcohol the procedure to be followed is given below.

The tissue was dipped in each grade of alcohol for about two minutes with two changes in the absolute alcohol. Trial helps to determine the time required for staining and differentation. Depending on the size of coverslip small amount of DPX was kept on the slide which should be enough to form thin film on the slide. The air bubbles were locked between the slide and coverslip were removed by leaving the slide overnight on a hot plate.

Xylene I (5 min)→Xylene II (5 min) →Propanol I (5 min) →Propanol II (5 min) →Alcohol I (5 min) →Alcohol II (5 min) →70% alcohol (5 min) →Haematoxylene (30 min) →Tap water (1-2 Dip) →Acidic alcohol (1-2 dip)—Tap water (1-2 dip) →70% alcohol(5 min)→Eosin(1-3 dip) → Absolute alcohol(5 min) →Propanol I (5 min) →Propanol II (5 min) →Xylene I (5 min) →Xylene II (5 min) →Xylene III (5 min)→mounted in DPX.

RESULTS

During the process of research various changes in the behaviour as well as external feature was observed directly with the eye where as histological changes were observed with the help of digital microscope. The various changes appeared in the body of fish in different conditions are categorised as behavioral and histological changes and explain below:-



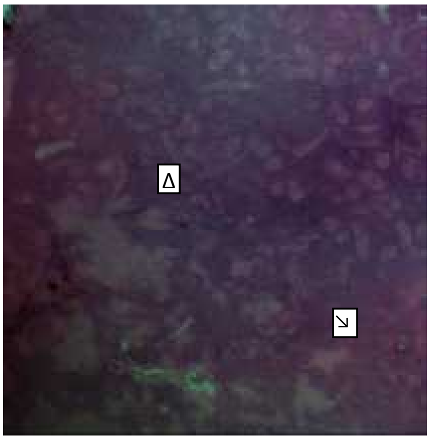


Fig:-2 and 3 showing the section of untreated fish kidney Glomerulus and Bowman's capsule (Δ) Distal tibule (↙)

Fish of control condition

After the aclimitization, fishes were kept in aquarium without treatment, so as to observe the normal condition of fish. Fish of aquarium without treatment showed normal behaviour so exhibited normal movement and feeding.

Study of histology of kidney of fish after sacrificing the animal and microtomy of tissue showed normal organization of cells. Trunk kidney of the control catfish, *Clarias batrachus* consisted mainly of haemopoeitic tissue, nephrons, convoluted tubules and collecting ducts. Each nephron possessed a renal capsule, a short neck and convoluted tubules. The renal capsule comprised glomerulus and Bowman's capsule. Glomerulus was formed by central rounded compact mass of numerous mesengial cells which was surrounded by the tufts of glomerular capillaries. Bowman's capsule contained thin squamous epithelium with an outer peritoneal and inner visceral layer. The proximal convoluted tubule consisted of cuboidal epithelial cells with basal nuclei, luminal surface being lined by well-developed brush border. The distal convoluted tubule comprised cuboidal epithelial cells which occupied only one third of the total tubule. The cytoplasm of the epithelial lining cells was slightly granular (Fig-2 & 3).

Fish treated with *Agave americana* (24 Hours)

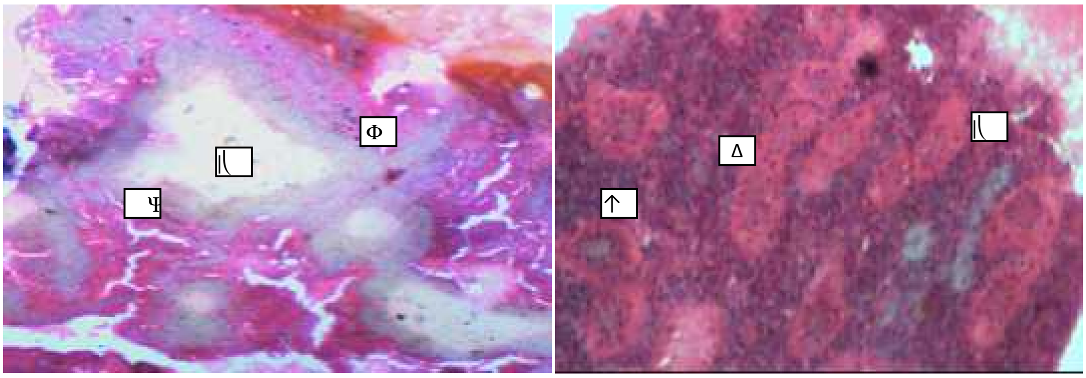


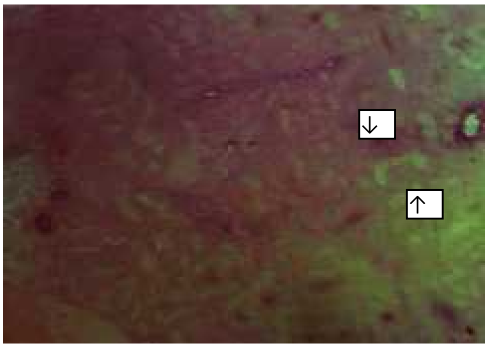
Fig:-4 & 5 showing the section of Treated kidney of 24 hours

Large Collecting duct (⟅) clumping of Melanomacrophages (Φ), Degenerated Pseudostratified epithelial cells (Ψ), Hypertrophic nucleus (Δ), Degeneration of renal tubules (↑), Renal epithelial cells (⟅).

The fishes treated with *Agave americana* showed difference in their behaviour then those of the control condition. Fish become restless and showed vigorous movement. Secretion of slime from the skin was increased, with reduced feeding from the normal which may be due to the loss of appetite.

The number of melanomacrophages increased and clumping of it started.Renal tubules started to degenerate. Pseudostratified epithelial cells also degenerated. Large collecting ducts were formed. Vacuolar/hydropic degeneration and fibrosis was appeared and tissue accumulation of the toxic was seen (fig:-4 & 5).

Fish Treared with *Agave americana* 48 hours



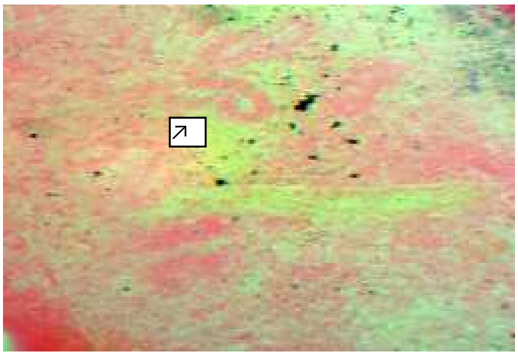
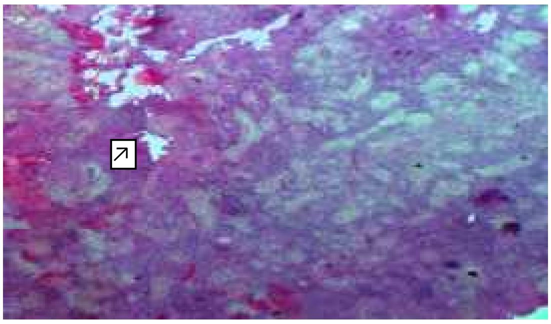


Fig:-6and 7 showing the section of treated kidney of 48 hours Detached epithelial cells of renal tubules (↗), Glomerular contraction clear (↑), Bowman’ s capsule(↓).

Fish was restless showing erratic movement and excess of secretion of slime on its body. They jumped vigorously and lost their balance. They stopped feeding.

The mild degenerative changes in the renal tubules started and epithelial cells was detached.Contraction of glomerulus was clear and Bowmans capsule space increased. Variable sized glomeruli appeared. Necrotic changes in the proximal convoluted tubule appeared (Fig:-6 and 7).

Fish Treated with *Agave americana* 72 hours



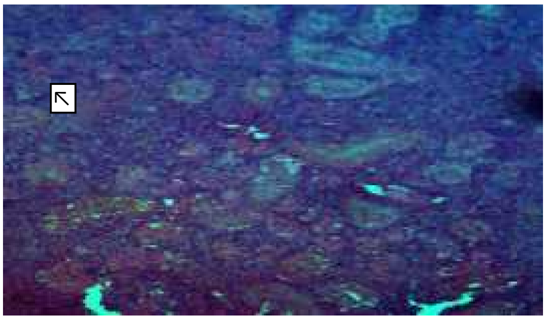


Fig:- 8 and 9 showing section of treated kidney of 72 hours Regeneration of renal tubules(↖), Vacuolization(↗)

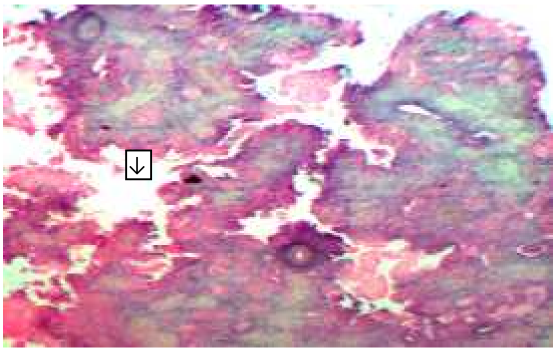


Fig:-10 showing section of treated kidney of 72 hours, Necrosis of tissue (↓),

Fish was slugish and showed lethargic movement. It stopped feeding, excessive secretion of sticky substance (slime) was appeared. It exhibit rapid oppercular movement which may be due to the difficulty in breathing.

Necrosis of the kidney tissue along with the regeneration of distal tubules appeared. Dilation of blood vessels observed. The tissue showed vacuolization. Increased granulation and hyperplasia of proximal convulated tubule was observed. The cloudy mass was observed, which indicates the deposition/accumulation of the toxic in the tissues (fig:-8, 9 & 10).

After 96 hours fish dies which may be due to some hormonal changes. For the accurate inference several studies like biochemical assay and electron microscopic study is needed. The cloudy mass was observed which inducates the deposition of the toxic in the tissues.

DISCUSSION

The present study revealed that 'Ketuke' exposure induced histopathological alterations in kidney of a freshwater catfish, *Clarias batrachus,* severity of the lesions was duration dependent. Under present investigation, it has been observed that the kidney of fishes exposed to sublethal dose of 'Ketuke' for 24, 48, 72 and 96 hours exhibited several histological alterations like, degeneration of epithelial tissues, clumping of melanomacrophages, glomerular contraction, and development of vacuoles in cell cytoplasm and necrosis of kidney tissue.

The kidney is a major site for toxic effects due to a wide variety of environmental pollutants (Foulkes and Hammond 1975, Hook 1980). Because of water reabsorption taking place in the distal tubules, relatively high concentrations of toxins may have an effect on renal cells.

The teleostean kidney is one of the first organs to be affected by contaminants in the water (Thophon *et* al. 2003) and it was seen in the present study.

According to Kent (1998) the liver and kidneys are involved in the detoxification and removal of toxic substances circulating in the blood stream.

Handy & Penrice (1993) found swollen Bowman´s capsule cells and melanomacrophages in the kidney of trout (*Salmo trutta*) and tilapia (*Oreochromis mossambicus*) exposed to mercuric chloride. Similar alterations were found in fishes exposed to organic contaminants (Veiga *et* al. 2002) and mixed environmental contaminants (Schwaiger *et al.*, 1997; Pacheco & Santos 2002), in the present study with cloudy swelling in tubule cells.

After 48 hours exposure, number of renal tubules was increased to overcome the toxicity. In fish the development of new nephrons continues throughout life, being more frequent in young and fast-growing fishes (Reimschuessel 2001). Hinton & Laurén (1990) and Cormier *et* al. (1995) have reported the increase in the frequency of new nephrons and regenerated tubules, during the process of the recovery of the damaged kidney in fish. Similar observations have also been made in the siluriform *Ameiurus nebulosus* and in cod (*Microgadus tomcod*) collected from contaminated streams (Cormier *et al.*, 1995), and in goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), zebrafish

(*Brachidanio rerio*) and tilapia (*Oreochromis mossambicus*) exposed to contaminants such as mercury, antibiotics and solvents, that are known to cause necrosis and vacuolization (Reimschuessel 2001).

Sharma and Gautam (1978) observed necrosis in glomeruli and tubules of sheep infected with experimental toxoplasmosis. The glomeruli were thickening with increase in glomerular space and condensation of Bowman's capsule.

The present observations are in agreement with the observations of Klei and Crowell (1981) in Brugia pahangi infected birds. George and Iyer (1981) observed variable sizes of glomeruli, degenerative changes in distal convoluted tubules of rats infected with Trichosomoides crassicaudo.

Pathological changes such as cloudy swelling, variable sized glomeruli, and degenerative changes in proximal convoluted tubules, distal convoluted tubules and Bowman's capsule, necrotic changes as well as edema were also observed in chick challenged with experimental *Ascaridia galli* infection. Necrosis is usually associated with an early loss of membrane integrity resulting in leakage of the cell content into immediate tissue environment and the induction of inflammation (Holle *et* al. 2003). Kobayashi *et* al. (2004) also reported necrosis and edema in fish infected with *Pseudomonas plecoglossicida*. Further, the necrotic cells showed karyorrhexis, akaryopyknosis and hyperchromatosis as well as cloudy swelling in infected kidney of (Sweetfish) *Plecoglossus altivelis*.

The kidney showed necrotic infection which appears to represent some sort of water and protein disturbances in the epithelial cells (George and Iyer 1981). After 48 hours, these changes were more pronounced showing necrosis in few proximal convoluted tubules, atrophy and shrinkage in glomeruli. A distinctive acute tubular epithelial necrosis associated with interstitial haemorrhages of the posterior kidney was originally described as haemorrhagic kidney syndrome in Atlantic salmon (Byrne et al. 1998).

Under present investigation, it has been observed that the kidney of fishes exposed to 72 hours exhibited several histological alterations like loosening, formation of clusters and lumps in haemopoietic tissue, vacuolization and degeneration of the cells of uriniferous tubules and shrinkage in glomeruli. Severity in alterations increased in proportion to increased dose and time period. Similar results have been reported by Cengiz (2006) in

Cyprinus carpio after acute exposure to deltamethrin. Giari *et* al. (2007) in European sea bass (*Dicentrachcus labrax)* after different cadmium concentrations exposure (4.47, 5.63, 7.08 and 8.91 mg/l) for 24 and 48 hours. Mishra and Banalata (2008) in *Channa punctatus* exposed to hexavalent chromium and Ahmad (2011) in *Clarias batrachus* exposed to 4 ppm and 8 ppm cadmium chloride for 90 days.

CONCLUSION

Exposure to sublethal concentrations of Ketuke (*Agave americana*), caused duration dependent histopathological alterations in the kidney of *Clarias batrachus*. The disorganization in this vital organ kidney might have resulted in physiologic and metabolic dysregulations, which further led to behavioral alterations and death of the species. In the longrun, therefore, Ketuke exposures to even sublethal concentrations may pose serious threat to fish health and affect their population if used hapazardly. Complementary studies are needed for further evaluation of this.

More drastic degenerative changes were seen at 48 hours since renal tubules were formed so less necrosis was seen at 72 hours. The experiment at 96 hours showed the death of fishes which indicates that there might be some biochemical/hormonal disorders. At the end of 96 hours fishes were very inactive indicating that it might have its effect on nervous system. Since its deposition was seen in the tissues, it might affect the higher groups of animals and as well as man because directly or indirectly it is their principal food.

RECOMMENDATIONS

There is a need of further research to define the roles of herbal pesticide in relation to human health when injested through fish indirectly. Very great care needs to be exercised in evaluating or acting on spedific claims of therapeutic benefit/harm from injesting phytochemicals via fish poison.

Isolation and identification of pescicidal substance from aquatic weeds is of great importance to reveal possible sources of environmental pollution and to find new biologically active substance for future practice use. Such work helps to effective use of abundant varities of plant resources.

Agave americana (Ketuke) known to have three main uses ornamental, medicinal and agricultural however, much is required to explore about it for the effective utilization in Nepal, Thus further research is recommended to have the best utilization of locally available plants like this.

I request to grant more encouragement at sub cellular and molecular studies of fishes as they are the integral part of human diet.

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