Effects of *Leucaena leucocephala* (Lam. de Wit) leaves and seeds on *Channa punctatus* (Bloch and Schneider) and *Oreochromis niloticus* (Linnaeus)

A

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Dedicated

To

My Beloved Parents

And

My Supervisor

RECOMMENDATION

The undersigned certify that this is a bona-fide research work of Tasmema Begum and the results which are embodied in this thesis are the investigation and works of her own for the degree of Doctor of Philosophy. This dissertation is suitable for submission.

(Prof. Dr. Gulshan Ara Latifa)
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DECLARATION

I am submitting my dissertation to the University of Dhaka for the fulfillment of the degree of Doctor of Philosophy (Ph.D). I do hereby declare that this submission is my own work and that to the best of my knowledge and belief it is not written by another person which has not been submitted for the award of any other degree. I have performed this research work under the supervision of Dr. Gulshan Ara Latifa, Professor, Department of Zoology, University of Dhaka.

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ABSTRACT

To investigate the effects on *Channa punctatus* (Bloch and Schneider) and *Oreochromis niloticus* (Linnaeus) after feeding *Leucaena leucocephala* (Lam. de Wit) leaf and seed for using as fish feed. The food was given at the rate of 2% of the body weight of fish. The observed amounts used in the bioassays were calculated in gm/kg body weight. The diet was formulated containing different levels of *L. leucocephala* leaf 25%, 30%, 35%, 40% and seed 20%, 25%, 30%, and 35% with small fishes. The experimental diets were given to the fishes divided into several groups on the basis of leaf and seed levels for two months. The effects of the *L. leucocephala* leaf and seed were considered as behavioral, physical, haematological and histopathological changes in *C. punctatus* and *O. niloticus.* The effects observed in behavior of *C. punctatus* and *O. niloticus* due to *L. leucocephala* leaf and seed were loss of appetite and slow movement. The physical changes of *C. punctatus* and *O. niloticus* due to *L. leucocephala* leaf and seed were observed i.e. lesions on skin and on caudal region, erosion of fins, puffed and loose scales, and exophthalmus. Haematological parameters (TEC, TLC, thrombocyte, lymphocyte, monocyte, eosinophil, basophil, Hb,Hct, MCV, MCH and MCHC) and biochemical parameters (protein, albumin, globulin, glucose, urea, creatinine and cholesterol) of *C. punctatus* and *O. niloticus* were determined after feeding *L. leucocephala* leaves and seeds. All the haematological parameters of 25% leaf group, 20% and 30% seed group of *C. punctatus* and 35% seed group of *O. niloticus* were positively correlated with control group. Positive correlation was observed in biochemical parameters of all groups of *C. punctatus* and *O. niloticus* compared with control group. In case of *L. leucocephala* leaf, lymphocyte and eosinophil, total protein, albumin, globulin were highly significant in *C. punctatus* whereas thrombocyte, lymphocyte, neotrophil, eosinophil, protein and albumin were highly significant in *O. niloticus* at the 5% level of significance. In case of *L. leucocephala* seed, albumin of *C. punctatus* and eosinophil of *O. niloticus* were highly significant at the 5% level of significance. The effects of feeding *L. leucocephala* leaf and seed on the number of eggs of *C. punctatus* and *O. niloticus* were highly significant at the 5% level of significance. The histopathological changes found in tissues i.e. eye, gill, muscle, stomach, intestine, liver, kidney and gonad of the experimental fishes. The effects were observed specially at higher percentages 35% and 40% of the *L. leucocephala* leaf and 30% and 35% of seed. The histopathological changes in testes and ovaries of *C punctatus* and *O. niloticus* in this experiment at 25%, 30%, 35%, 40% *L. leucocephala* leaf and 20%, 25%, 30% and 35% seed groups showed the decreasing trend of tertiary oocyte, spermatid and spermatozoa with the increasing of percentages of *L. leucocephala* leaf and seed level.

INTRODUCTION

Fish is a good source of animal protein. The role of fish in the life of the people is unlikely to diminish and nutritionists believe that better health of the people can be ensured quickly and economically through greater production of fish. Fish lives in a very intimate contact with their environment they are extremely dependent on it. Any changes in the environment would affect the physiology of the fishes.

The fresh water fish “Zeol fish” offers a unique and abundant commercial potentiality because of their accessory respiratory organ. They possess the unique capability of surviving under extreme environmental condition. The fresh water air breathing zeol fish *Channa puntatus* (Bloch and Schneider, 1801) locally known as lata fish. They are predatory fish distributed throughout Bangladesh, India, Srilanka, Pakistan, Burma, China, Malaysia, Thailand, Korea, Vietnam, Kampuchea and Philippines. They belong to the family Channidae, order Channiformes. In Bangladesh 5 species are available. All of them known as “Murrel”. They are also known as “Snake headed” fish. They are best suited to shallow water in ponds and are also inhabitant in river, beel, haor, canal etc. The snakehead fish production is about 89,351 metric ton per year, which is the 3.33% of the total production (DOF 2011-12). They can be cultured in derelict pond. In view of commercial importance of these fishes it is very necessary to take measures of proper scientific management and harvesting.

Oreochromis niloticus is an exotic fish native to Africa. Tilapia are mainly freshwater fish, inhabiting shallow streams, ponds, rivers and lakes, and less commonly found living in brackish water. Historically, they have been of major importance in artisan fishing in Africa and the Levant, and are of increasing importance in aquaculture. *Oreochromis niloticus*, the Nile tilapia, is a relatively large cichlid fish, belongs to the family Cichlidae, order Perciformes. It is a plankton feeder, an omnivore, and to feed on higher plants to the extent it may be used in control of aquatic weeds.

Haematological parameters have a significant meaning in the field of disease diagnosis.The blood of tilapia and other fish, as in the case of other vertebrates, can be defined in general terms as a liquid in which the principal cellular elements (erythrocytes and leucocytes) are suspended in an isotonic plasma.

Medical terms related to blood often begin with ***hemo-*** or ***hemato-***(also spelled***haemo-*** and ***haemato-***) from the Greek word αἷμα (*haima*) for "blood". In terms of anatomy and histology, blood is considered a specialized form of connective tissue, given its origin in the bones and the presence of potential molecular fibers in the form of fibrinogen. It is a specialized bodily fluid in animals that delivers necessary substances such as nutrients and oxygen to the cells and transports metabolic waste products away from those same cells.

Fish plasma is a clear opaque liquid in which, are found a part from the blood cells, dissolved minerals, absorbed products of digestion, waste products of the tissues, special secretion, enzymes, antibodies and dissolved gases. Fish have low levels of plasma proteins. The major proteins of fish blood plasma are globulins, to a large extent antibodies – immunoglobulin. The blood volume of fishes varies between 2 and 8% of the body volume (Fange, 1994). One third to one half of the total blood volume in fish consists of blood cells (Wedemeyer *et.al.* 1977), the rest is fluid plasma.

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. Histological studies may be conducted via tissue culture, where live cells can be isolated and maintained in a proper environment outside the body for various research projects. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Histology is an essential tool of biology and medicine.

Histopathology*,* the microscopic study of diseased tissue, is an important tool in anatomical pathology, since accurate diagnosis of cancer and other diseases usually requires histopathological examination of samples.

The study of histopthalogical effects of various pollutants, toxic materials of plant parts Organo- phosphates, and insecticides on the different organs of fishes are important basic effort leading to our understanding of true impact of pollutants and others on the ecosystem because the freshwater fishes show dissimilar pattern of responses when exposed to various pollutants. (Gardner and Laroche 1973; Vijayamadhawan and lwai 1975) reported that the extent of damages vary with body part, nature of pollutants, medium and duration of tests.

Available literature mostly deals with laboratory and field bioassay tests attempting to determine the percentage mortality and LC_{50} (kabir and Ahmed, 1979). Literature citations on the toxic effect of the plant preparations on different organs of fishes "specially on the histopatholgy of organ" were very limited and the available ones mostly deal with environmental pollutants and the salts of metals. Aktar (1994) studied histopatholgical changes in gill, liver, kidney, stomach and intestine after '8' hours exposing of *H. fossilis* to 50% ethyl alcohol extract of dry root bark of *Barringtonia acutangula* (Gaertn). Matton and La Ham (1969) reported histopathogoical changes in the heart, liver, psueudogills, and muscle fibres of rainbow trout larva treated for 16 hours with Dylox at 10-100 ppm. Eller (1971) studies histopatholgical changes in liver, gill brain, pancreas and gonad after chronically exposing *Salmo clarki* to endrin by bath or in food. Sivarajah *et.al*. (1978) worked some histopatholgical effect of Aroclor (PCB) on liver and gonads of rainbow trout and carps. De and Datta (1990) studies on certain aspects of the morphohistology of Indian shad Hilsha, *Tenualosa ilisha* (Hamilton) in relation to food and feeding habits.

To observe the changes in the various organs of the affected fish it was necessary to obtain a detailed information of the normal (control) fishes. Until now a limited work have been carried out on the histopathology of the fishes.

The concept of feeding fish is the same as that applied in feeding other food animals; to nourish the animal to the desired level or form of productivity as profitable as possible. The nutrients required by fish for growth, reproduction and other normal physiological function are similar to those of land animals. They need protein, minerals, vitamins and growth factors, and energy sources. These nutrients may

come from natural aquatic organisms or from prepared feeds. If fish are held in an artificial confinement where natural feeds are absent, such as raceways, their feed must be nutritionally complete. However, where natural food is available, there supplemental feed is fed for additional growth the feed may not need to contain all of the essential nutrients (Lovell 1979).

Fishes evolved in an aqueous environment where carbohydrates were scarce, their digestive and metabolic system seem to be better adapted to utilization of protein and lipids for energy than carbohydrates. Some fishes, such as warm- water herbivores or omnivores, can digest and metabolize carbohydrates relatively well (Lovell 1984). Channid fishes are carnivorous and predatory by nature. They feed small fishes and subsist on a variety of living creatures including fish, frogs, snakes, insects, earthworms and tadpoles. So Channid fishes need protein enriched food. As animal protein is expensive, so we need to find out such plant protein, which can be supplemented for their protein requirement.

When fish are cultured in a system where natural foods are absent such as trout raceways or where natural foods make only a small contribution to the nutrition of the fish, as in intensively stocked catfish ponds, the feed should be nutritionally complete. However, where abundant natural food is available, supplemental feeds need not contain all of the essential nutrients. Nutritionally incomplete feeds are usually specific for a fish species or culture system. Management, environmental factors and fish size can have an effect on dietary nutrient levels for optimum performance. However, nutrient requirement data that are available serve relatively well as a basis to formulate highly productive, economical diets for commercial aquaculture.

Ingredients used in practical fish feeds can be classed as protein (amino acid) sources, energy sources, essential lipid sources, vitamin supplements, mineral supplements and special ingredients to enhance growth, pigmentation or sexual development in the fish or physical properties, palatability or preservation of the feed (Lovell 1981). A feed ingredient, which used worldwide and factors influencing that use in fish feed is discussed in the following.

The Plant *Leucaena leucocephala*

Taxonomic Name: *Leucaena leucocephala* (Lam. de Wit)

Synonyms: *Acacia leucocephala* (Lamark) Link 1822, *Leucaena glabrata* Rose 1897, *Leucaena glauca* (L.) Benth. 1842, *Mimosa leucocephala* Lamark 1783

Common Names: wild tamarind, ipil-ipil, lead tree, leucaena, subabul, tangantangan. In Bangladesh it is popularly known as Ipil-Ipil or Telikadam.

Description: The genus *Leucaena* is distinguished from all other Mimosoid legumes by its hairy anthers which are easily visible with a hand lens. *Leucaena leucocephala* is distinguished from other species of *Leucaena* by its intermediate leaflets and large pods in clusters of 5-20 per flower head. It forms a small to medium-sized thornless tree 3-20m tall and 5-50cm bole diameter

During the 1970s and early 1980s, *Leucaena leucocephala* (Lam. de Wit) was known as the 'miracle tree' because of its worldwide success as a long-lived and highly nutritious forage tree, and its great variety of other uses. As well as forage, *leucaena* can provide firewood, timber, human food, green manure, shade and erosion control. It is estimated to cover 2-5 million ha worldwide (Brewbaker and Sorensson 1990). However, a better understanding of its constraints, particularly the arrival of the psyllid insect, has now given us a more balanced view of the value of this species.

Leucaena has its origins in Central America and the Yucatan Peninsula of Mexico where its fodder value was recognized over 400 years ago by the Spanish conquistadores who carried leucaena feed and seed on their galleons to the Philippines to feed their stock (Brewbaker *et al.* 1985). From there it has spread to most countries of the tropical world where leucaena was used as a shade plant for plantation crops. It was introduced into Australia in the late 19th century and it was naturalised in parts of northern Australia by 1920 (White 1937).

The *Leucaena leucocephala* tree has been introduced in Bangladesh in the late 60's. After introduction massive propaganda have been made in favor of this tree to plant in different areas of the country depicting its utilities and usage. With this propaganda this tree has been planted in different areas of the country as roadside

tree, as decorative tree in the household garden, as a source of fodder, as a source of soft tree, as a source of firewood and for some other purposes.

In Bangladesh, the *Leucaena leucocephala* tree mainly used as fodder, as are being used in other parts of the world. The dairy and cattle, poultry and duckery, and fish in ponds have been supplied with the leaves of this tree. The leaves of this tree are used as a component of the fishmeal, or as the food of other animals. *L. leucocephala* (ipil-ipil) has been found to be a valuable forage and feed for our livestocks (Rahman and Dewan, 1988). Its leaf and seed meals are in wider use in many tropical countries including Bangladesh. Many reports have discussed its side effects in different domesticated animals and chicken. Rahman and Dewan, 1988 described pathological effects of *Leucaena* seed meal in the duck *(Anas platythyncha domestica)* in Bangladesh.

Leucaena foliage (leaflets plus stems) contain both nutrients and roughage, and make an almost complete ruminant feed, somewhat comparable to alfalfa forage (D'Mello *et al.* 1977).Its amino acid pattern is comparable with that of soybean and fishmeal (Ter Meulen *et al.* 1979) and other animal feed sources available in developing nations (D'Mello *et al.* 1977 and Kale, 1987).

The various parts of the plant, seeds and immature leaves contain the highest amount of crude protein, and the stem and dry pods the lowest (Kale, 1987; Yadav PS and Yadav IS,1988; Akbar and Gupta, 1985; Hilal *et al.* 1991). Leaf protein concentrate (LPC) was prepared from *L. leucocephala* leaf meal (LLM). The recovery of LPC was 7.6% and it contained 65.9% crude protein, compared with 29.2% in LLM. Ash content was 17.6%, and the levels of lysine, histidine, arginine, isoleucine, and leucine were 5.6%, 2.3%, 5.9%, 5.4%, and 11% on a dry matter basis, respectively. Glutamic acid, aspartic acid, leucine, and isoleucine are the major amino acids in the plant (Hilal *et al.* 1991). Total carbohydrate (18.6%), reducing sugars (4.2%), oligosaccharide sucrose (1.2%), raffinose (0.6%), stachyose (by difference) (1.0%), total oligosaccharides (2.8%), and starch (1%) have been reported (Kale, 1987).

The seeds are ovoid in shape and have brown hulls and yellow kernels. The hull: kernel ratio is 50:50 by weight (Sethi, 1989), although others reported a 60.5 : 39.5 ratio (Hossain, 1988). The seed is low in oil, 5.1% to 10% (Chandrasekhara Rao *et al.* 1984; Jagan Mohan Rao and Azeemoddin *et al.*1988; Kale, 1987;Chowdhury *et al.* 1984; Verma and Chandra, 1979; Jones and Earle, 1966; Kafuku and Hata, 1934), and rich in protein, 24.5% to 46% (Chandrasekhara Rao *et al.* 1984; Jagan Mohan Rao and Azeemoddin *et al.*1988; Ranjhan,1977; Ter Meulen *et al.* 1979; Kale, 1987; Yadav PS and Yadav IS,1988; Akbar and Gupta, 1985; Chowdbury *et al.* 1984; Verma and Chandra, 1979; Jones and Earle, 1966; Felker and Bandurski, 1977). The kernels have an oil content of 11.9% (Hossain *et al.* 1988;) to 15.3% (Azeemoddin *et al.*1988; Hossain *et al.* 1988; Felker and Bandurski, 1977) and a protein content of 52.5% to 66.4% (Azeemoddin *et al.*1988; Hossain *et al.* 1988; Padilla and Soliven 1933; Padmavathy and Shobha, 1987).Thus the nutrients are concentrated in the kernels (Azeemoddin *et al.*1988; Hossain *et al.* 1988; Padilla and Soliven 1933; Padmavathy and Shobha, 1987), which can be used as a concentrate for dairy animals (Ranjhan,1977).

It is significant that the proteins of *L. leucocephala* (Azeemoddin *et al.*1988; Ter Meulen *et al.* 1979; Kale, 1987; Felker and Bandurski, 1977), *L. pulverulenta*, and *L. macrocarpa* (Jones and Earle, 1966) seeds are fairly rich in the essential amino acids isoleucine, leucine, phenylalanine, and histidine. Lysine and methionine are also present in moderate amounts. The in vitro digestibility of *Leucaena* protein isolate (LPI) is 76% (Sethi and Kulkarni, 1993). Of the total free amino acids in *L. leucocephala* seed, 60% is mimosine (Sethi and Kulkarni, 1994).

Leucaena seeds have a total carbohydrate content of approximately 35 % to 45 % (Kale, 1987; Verma and Chandra, 1979; Sotelo *et al.*1980), with reducing sugars constituting 5.2% (Kale, 1987). Starch is absent from a number of the species (Sethi1989; Jones and Earle, 1966), including *L. leucocephala*, although it was reportedly 1.3% in the seeds (Kale, 1987). The total oligosaccharide content of *L. leucocephala* is 3.5% to 3.6% (Kale, 1987; Ford, 1979), with sucrose 1.9% to 2.0%, raffinose 0.7% to 0.8%, and stachyose 0.7% to 0.8% (Kale, 1987; Ford, 1979; Herissey and Mascre 1941). D. Pinitol (Plouvier, 1962) and myoinositol have also been found in seeds (Beveridge *et al.*, 1977).

The seeds contain a dark, green to brown, fatty oil with 5.1% to 10% yield (Chandrasekhara Rao *et al.* 1984; Jagan Mohan Rao and Azeemoddin *et al.*1988; Kale, 1987;Chowdhury *et al.* 1984; Verma and Chandra, 1979; Jones and Earle, 1966; Kafuku and Hata, 1934; Kulkarni *et al.*1992) containing approximately 26% to 29% saturated acids and 71% to 73% unsaturated acids (Kale, 1987; Kafuku and Hata, 1934). The oil is rich in linoleic acid (42.5-65%) (Chandrasekhara Rao *et al.* 1984; Kale, 1987; Hossain *et al.* 1988; Chowdhury *et al.* 1984; Kafuku and Hata, 1934; Mitsuhashi *et al.* 1972; Khalid *et al.*1989; Agrawal *et al.*1988;Ahmed *et al.*1988; Majumder and Chowdhury 1987) and contains significant quantities of arachidic (0.8-1.6%) (Chandrasekhara Rao *et al.* 1984; Hossain *et al.* 1988; Mitsuhashi *et al.* 1972; Khalid *et al.*1989; Agrawal *et al.*1988;Ahmed *et al.*1988; Majumder and Chowdhury 1987) and lignoceric acids (0.71.7%) (Chandrasekhara Rao *et al.*1984; Raval *et al.*1988). The principal constituents of the unsaponifiable fraction of the oil are sterol 35%, methylsterols 8%, triterpenoid alcohols 20%, tocopherols 17%, and total hydrocarbons and carotenoids 20% (Miralles 1982). The main sterol is sitosterol, 55% (Verma and Chandra, 1979; Kafuku and Hata, 1934; Mitsuhashi *et al.* 1972; Miralles 1982; Ford 1979), and the main tocopherol is □-tocopherol (Miralles 1982). The glycolipid composition of subabul seed oil was studied (Kulkarni *et al.*1992). The oil is the richest vegetable source of phosphatides (Jagan Mohan Rao and Azeemoddin 1988).

Leucaena seed has high calcium and phosphorus levels (Kale, 1987; Padmavathy and Shobha, 1987). Oxalic acid has been detected in these seeds (Sethi, 1989). Various vitamins, such as thiamine, riboflavin, niacin, betacarotenes, and ascorbic acid, are present in the hulled meal of *Leucaena* seeds a traditional food in Mexico (Rushkin, 1984).

Among the anti-nutritional factors, the alkaloid mimosine, beta-N (3-hydroxy-4-pyridone)- x-amino propionic acid, a toxic, non-protein amino acid (Ter Meulen *et al.* 1979), is an important constituent. The values for mimosine vary among the different species of *Leucaena* (Kewalramani *et al.* 1987; Sutikno *et al.* 1991). Mature seeds are twice as rich in mimosine as young seeds, 6.2% and 3.2%, respectively, but the reverse is true of the leaves, 2.6% versus 5.1% (Yadav PS and Yadav IS,1988; Akbar and Gupta,1984; Hilal *et al.* 1991; Adeneye 1991). Mimosine, contributes as

much as 14.8% to the total nitrogen content of *L. leucocephala* seeds. The mimosine content varies from 2.2% to 10% (Ter Meulen *et al.* 1979; Kale, 1987; Hongo *et al.*1987; Akbar and Gupta, 1984; Sethi 1989). The concentration of mimosine in the seeds varies widely among the various cultivars of the species (Sethi and Kulkarni, 1994).

Mimosine may exert its toxic action by blocking the metabolic pathways of aromatic amino acids and tryptophan (Lin *et al.* 1965). Due to its structural resemblance to Ltyrosine, it probably acts as a tyrosine analogue or antagonist that inhibits protein biosynthesis in the living body and causes toxic symptoms, including growth retardation (Lin *et al.* 1964; Ter Meulen *et al.* 1981; Serrano *et al.* 1983). The growth retardation of cattle consuming *Leucaena* is associated with lower serum thyrosine levels (Jones and Winter 1979-80), which in turn could be related to the reduction in serum tyrosine level caused by mimosine (El-Harith *et al.* 1981).

Usually fish farming used the extensive methods where the animals obtain all their nutrition from the aquatic environments in which they were cultured. By early in the twentieth century, the culture techniques have progressed to stocking fish at densities higher than could be supported by the natural productivity. The nutrients input into the culture systems were limited to fertilizers and crude agriculture and animal products or by-products. Recently, in response to the increased cost of land and labor, as well as increased demand for fish in the world market, fish husbandry has changed its form from extensive to semi-intensive or intensive systems. These latter employ modern facilities, equipment and technology to obtain significantly higher yield per unit area. High production levels depend on good nutrition as well as proper culture management. The quality and quantity if feed used are the major factors in determining profitability because feed represents the largest single expenditure in semi-intensive or intensive culture operations. Thus, economical production depends on availability of least-cost, nutritionally-balanced diets (Chhorn Lim and Warren Dominiy 1989).

Unlike domesticated farm animals, fish species currently cultured have a high dietary protein requirement. Consequently, only high protein content feedstuffs are included in fish feeds. Fishmeal has traditionally been used as a major protein source because of its high nutritive value and palatability. Recently, the high cost and short

supply of fish meal has made it necessary to substitute with cheaper plant proteins such as soybean meal, peanut meal, cottonseed meal, sunflower seed meal, rapeseed meal and *Leucaena* leaf meal in order to sustain economical fish production. Leaf meal of *Leucaena Leucocephala*, which contains approximately 29% crude protein on a dry matter basis has been used in the tropics as a protein source in ruminant and poultry feeds. However, its use has been limited because of the presence of the toxic non-protein amino acid, mimosine (Chhorn Lim and Warren Dominiy 1989).

A few studies have been conducted to evaluate the nutritive value of *Leucaena Leucocephala* leaf meal as a protein source in fish feeds, but the data obtained are conflicting. Pantastico and Baldia (1979, 1980) reported improved growth responses of *Tilapia mossambica* and *T. nilotica* fed diets containing 100% *Leucaena* leaf meal. However, Jackson *et. al.* (1982) obtained very poor growth of *S. mossambicus* fed diets in which 25% of the fish meal was replaced by *Leucaena* leaf meal. This growth reduction was attributed to the toxic effects of mimosine. A trend of reduced growth performance and feed efficiency with increased dietary levels of *Leucaena* leaf meal was also reported by Wee and Wang (1987) for nile tilapia. The reproductive performance and growth of *O. niloticus* broodstock were also affected by the presence of high levels of *Leucaena* leaf meal in diets. Santiago *et. al.* (1988) recommended that *Leucaena* leaf meal should not be incorporated to more than 40% of the diet of nile tilapia broodstock.

Fishmeal is customarily used as a major protein source in fish feeds because of its high nutritive value and palatability. To date, fishmeal still constitutes a substantial part of the feed formula for aquacultural species. However, the rising cost and uncertain availability of fish meal have forced aquaculture nutritionists and feed manufacturers to use less expensive, readily available plant protein as a substitute for fish meal (Chhorn Lim and Warren Dominiy 1989).

Keeping the information of the *Leucaena leucocephala* in mind the present research work to observe the effects of *L. leucocephala* on two species of fish has been proposed.

The aims and objectives of the present work

I. Investigating the chemical composition of the leaves and seeds of the *Leucaena leucocephala* (Lam. de Wit)

II. Evaluating the usages of leaves and seeds of *L. leucocephala* as fodder of fish.

III. To work out the safety dose ranges on the species studied.

IV. Observing the behavioral cshanges of *Channa punctatus* and *Oreochromis niloticus* fed different amount of *L. leucocephala* leaves and seeds.

V. Observing the physical changes of *C. punctatus* and *O. niloticus* fed different amount of *L. leucocephala* leaves and seeds.

VI. Evaluating the changes in haematological parameters (TEC, TLC and differential count, hemoglobin, hematocrit, MCV, MCH, MCHC) caused due to feeding of *L. leucocephala* leaf and seed.

VII. Evaluating the changes in blood serum parameters i.e. total protein, globulin, albumin, glucose, urea, creatinine and cholesterol.

VIII. Histopathological changes of the vital organs, i.e. tissues of eye, gill, muscle, stomach, intestine, liver, kidney and gonad of *Channa punctatus* and *Oreochromis niloticus*.

REVIEW OF LITERATURE

The literature reviewed here includes some earlier works, which has relevance to the present studies.

Kakkar P. H. *et. al*. 2011, observedthe effects of water soluble fraction (WSF) of diesel fuel in liver of *Channa punctatus*. The fishes were exposed to sublethal concentration of WSF of diesel (5%-DF1, 10%-DF2, 15%-DF3, 20%-DF4 and 25%-DF5) for 21 days. Significant histopathological lesions observed were dilation, congestion, thrombosis formation in hepatoportal blood vessel, melanomacrophage centers, hemolysis, hemorrhage, lymphocytic infiltration between the hepatocytes and necrosis & fibrosis in hepatocytes were the prominent changes in liver. The histological analysis showed increasing damages dose-dependents and time-dependents.

Ghosh R. *et. al*. 2011, observed the histopathological changes in head kidney and liver, erythropoietic and leucopoietic cells in head kidney and total erythrocytic and leucocytic populations in the peripheral blood of adult *Channa punctatus* (Bloch) following experimental inoculation of sublethal dose of *Aeromonas hydrophila* and *Aeromonas salmonicida* respectively during various exposure periods. Histopathological examinations showed initiation of necrosis, periglomerular degeneration in head kidney and formation of necrotic hepatocytes in liver compared to sham-injected control (SIC). Significant changes in total erythrocytic count (TEC) in aeromonad-treated groups were less pronounced over the most exposure periods. Similar observations were also recorded from the study among erythropoietic cell lineage from the head kidney tissue imprints. On the contrary, neutrophils, lymphocytes and macrophages among the leucocytes elicited significant variations in aeromonad-injected fish throughout the exposure periods with little fluctuations in the head kidney. An initial steep increase in the total leucocytic count (TLC) was also found in the peripheral blood of aeromonad-treated groups.

Jagtap A. R. *et. al*. 2011, studied thetoxic influence of metals produces physiological changes in the organ of animal. The different haematological tests were carried out on a fresh water fish*, Channa punctatus* after exposed to sublethal

concentrations of copper sulphate. The haematological studies have long been cosidered as a valuable diagnostic tool in clinical biochemistry, population, genetics and in medical anthropology. The haematological tests like RBc Counting, WBC Counting, Haemoglobin Content and ESR Value were carried out on the fish exposed to copper sulphate upto 96 hours. The values of RBC Count, Haemoglobin Content were found to have decreased. However, the WBC Count, ESR registered a marked increase as compared to control.

Ada F. B. *et. al*. 2011, observed the Nile Tilapia, *Oreochromis niloticus* was exposed to Paraquat for 24 h so as to monitor its effects on the fish's haematology, and general behaviors. The haematological parameters estimated include haemoglobin, mean cell haemoglobin, mean cell haemoglobin concentration, packed cell volume, mean cell volume, erythrocyte sedimentation rate and white and red blood cell counts. The hepatosomatic index was measured for different concentrations of Paraquat exposed. Results showed that the hepatosomatic index was decreasing with increasing concentration of Paraquat. Haemoglobin, mean cell haemoglobin, mean cell haemoglobin concentration and erythrocyte sedimentation rate were observed to be negatively related to concentration of Paraquat. Packed cell volume, white blood cell count (WBC), red blood cell count (RBC) showed positive relationship with concentration while mean cell volume was not significantly changing with change in concentration of Paraquat.

Palipoch S. *et. al*. 2011, observed that the *Thunbergia laurifolia* leaf was extracted by using 50% ethanol and supplemented with normal fish food. *Oreochromis niloticus* (n = 120) were divided into 6 groups by treating with or without 45 ppm ofwaterborne lead (II) nitrate and feeding normal fish food, fish food supplemented with *T. laurifolia* leafextract in low or high dose. After 28 days of treatment, peripheral blood and organs were collected. Growth rate, blood chemistry, hematology and histology were investigated. Fish which were exposed to $Pb(NO_3)_2$ and were fed with fish food supplemented with *T. laurifolia* leaf extract especially in high dose, exhibited higher specific growth rate than fish which were exposed to $Pb(NO_3)_2$ and were fed with normal fish food. Moreover, *T. laurifolia* leaf extract can normalized blood chemistry, hematological and histological parameters in Pb(NO3)2-treated *O.*

niloticus. We conclude that *T. laurifolia* leaf clearly reduced toxicity and is able to promote growth performance in *O. niloticus* after Pb(NO3)2 exposure.

Ashish K. Mishra and Banalata Mohanty, 2009, studied the effects of chronic exposures (one and two months) to sublethal doses of hexavalent chromium (2 and 4 mg/L potassium dichromate) on organ histopathology and serum cortisol profile were investigated and their overall impact on growth and behavior of a teleost fish, *Channa punctatus* was elucidated. Histopathological lesions were distinct in the vital organs gill, kidney and liver. The gill lamellae became lifted, fused, and showed oedema. Hyperplasia and hypertrophy of lamellar epithelial cells were distinct with desquamation. Hypertrophy of epithelial cells of renal tubules and reduction in tubular lumens were observed in the trunk kidney. The atrophy of the head kidney interrenal cells and decreased serum cortisol level indicated exhaustion of interrenal activity. Hepatocyte vacuolization and shrinkage, nuclear pyknosis and increase of sinusoidal spaces were observed in the liver. Abnormal behavioral patterns and reduced growth rate were also noticed in the exposed fish. The chronic hexavalent chromium exposure thus by affecting histopathology of gill, kidney (including interrenal tissue) and liver could impair the vital functions of respiration, excretion, metabolic regulation and maintenance of stress homeostasis which in the long-run may pose serious threat to fish health and affect their population.

Atif M. El-Naggar, 2009, collected fish samples from the River Nile and its two branches (Damietta and Rossetta).The sampling stations were El-Oqsur, El-Menia, El-Hawamdia, Shoubra El-Khema, El-Rahawy drain, KomHamada, Talkha, El-Serw and Faraskour. The accumulation of Iron, Manganese, Copper, Zinc, Cadmium andlead in liver of collected *O. niloticus* fish from the above stations were determined. In addition, the sameliver samples were examined histopathologically. Results showed that trace metals accumulations in fish liverat area under investigation were detected in following descending order: Fe > Cu > Zn > Mn > Pb > Cd. Histological study indicated that the liver of *O. niloticus* living in the studied stations showed severalpathological alterations including: degeneration, fatty degeneration, necrosis and edema. Also congestion, branching (anastimosis), hemorrhage, hemolysis, hemosidrin and parasitic forms were seen in blood

vessels.It was noticed that the liver of fish collected from Shoubra El-Khema, El-Hawamdia, El-Rahawy drain and El-Menia showed much more damages than that collected from the other stations as these sites receives moredrainage water loaded with industrial and sewage wastes than the other stations. It was concluded that the discharge of different types of wastes, especially heavy metals deteriorated the water quality in the River Nileand consequently affecting fauna and fish production and human health. It is recommended to treat the different wastes before discharging to the River Nile Stream.

Zaki M. S., 2008, observed the present study was planned to investigate the effect of *Saprolegnia parasitica* infection in then hematological, serum biochemical and pathological alterations of *Oreochromis niloticus*. Forty five fish were divided into three equal groups. Fish of first group served as a control. Fish of groups (2&3) were infected by *Saprolegnia parasitica*. Fish of group (3) were treated after 7 days of post-infection using potassium permanganate for 10 days. Sampling was done after 1 and 7 days of post-infection (gps 1 & 2) and 10 days of post-treatment (gps 1 & 3). The results revealed a non significant changes in the hematological and the biochemical parameters after 1day of infection, but after 7 days of post- infection and 10 days of post-treatment, a significant decrease in RBCs , Hb, PCV and significant increase in AST, ALT, urea, creatinine , sodium, potassium, cortisol, insulin and glucose were seen. Iron showed a significant decrease at the same period of sampling. The pathological examination revealed a massive fungal growth resembling a tuft of cotton wool threads was seen in eyes, gills, fins and in localized areas of the skin. Microscopically, the fungal hyphae and spores appeared on eyes, gills, skin and underlying muscles with marked degenerative, necrotic and inflammatory reactions. These reactions were evident, after 7 days of post- infection and the severity of the lesions were markedly decreased after 10 days of post-treatment. It could be concluded that, saprolegnia parasitica infections induced marked tissue alterations as well as some hematological and serum biochemical changes. Although potassium permanganate treated the infected cases and allowed the regenerative processes but it does not progress the hematological and serum biochemical parameters.

Mishra A. K. and Mohanty B.2008, studied the histopathological effects of hexavalent chromium (Cr VI) in the ovary of a fresh water teleost, Channa punctatus were investigated. An exposure-dependent alteration in ovarian histology is reported. For both acute and chronic exposures to Cr (VI), the percentages of atretic oocytes were increased; this increase was more pronounced in the acute exposure group. A decrease in percentage of vitellogenic oocytes was observed in the chronic exposure group indicating impairment of vitellogenesis. The hepatocellular vacuolization and atrophy along with pyknotic nuclei in both acute and chronic chromium exposed fish liver supports the vitellogenic impairment. The observed alterations may be due to both direct cytotoxic effect of Cr (VI) on the ovary as well as mediation by overall systemic toxicity affecting other vital organs.

Gautam, R. K.and Kumar S., 2008, deals with the haematological changes in fresh water snake headed fish *Channa punctatus* after exposure to the sublethal concentration of organophosphate Nuvan [dichlorvos]. The haematological observations at the sublethal toxicity of Nuvan showed a decrease in the number of total erythrocyte count (TEC), haemoglobin concentration (Hb%) and packed cell volume (PCV). The total leukocyte count show comparison to non treated fishes.

Sotolu A. O. 2008, observed high cost of fishmeal for fish feeds is a major constraint to the growth of aquaculture in Nigeria. Dearth of information on suitable alternatives to fish feed ingredients persists. Processing and digestibility potentials of *Leucaena leucocephala* seed meals (LSM) as feed ingredient were therefore investigated in *Clarias gariepinus* diets. The seeds were collected and processed by sun-drying (SD), toasting (TS), soakingin water (SW) and soaking in alkaline solution (SA). Four isonitrogenous diets {40% Crude Protein (CP)} were prepared using the processed LSMs and a 14-day digestibility study was conducted using *C. gariepinus* fingerlings. *C. gariepinus* fingerlings (average weight 5.72±0.02g) were subjected to treatments in three replicates using a Completely Randomized Design. Chemical analyses of experimental feeds and fish were conducted before and after feeding trials. Dissolved oxygen, temperature and pH were monitored and data were analysed using descriptive statistics, ANOVA and correlation. Differently processed LSMs varied generally both in their nutrient and mineral compositions. SW produced meal of highest CP (36.01%) with highest Mean Weight Gain (MWG) of 0.32g while SD

had the least CP (22.75%) and the least MWG. Digestibility coefficients of protein and calorie varied significantly ($p<0.05$) among fish-fed processed LSM from 66.9 (SD) to 73.6 (SW)and 64.2 (SD) to 70.2 (SW) respectively. Fish carcass protein was only marginally different among treatments with the highest (63.34%) in SW and the least (62.11%) in TS. Survival rate was generally high in all treatments with overall mean of 95%. *Leucaena leucocephala* offers a good potential as a cheaper plant protein sourcein fish diet with high nutritive value. *Clarias gariepinus* was able to utilize SW-based diet better than other processed *leucaena* for sustainable aquaculture.

Mishra, D. K.*et. al*. 2008, recorded the histopathological alterations in gill, kidney and liver induced by sublethal exposure (30 per cent of LC_{50} for 96 hours) to Carbaryl (5.20 mg l^{-1}) and Cartap (0.18 mg l^{-1}), *Channa punctatus* were given treatments for 24, 48, 72 and 96 hours under static bioassay conditions. Gills responded initially with profuse secretion of mucus filling the interlamellar spaces and swelling of the tips of the secondary gill lamellae in both the treatments. At places, initial separation of the epithelial lining cells from the pillar cells were also noticed. At 48 hours, oedematous separation of epithelial lining cells from the pillar cells were more pronounced. Hyperplasia in the secondary lamellae was observed in the gills at 72 hours whereas complete fusion of secondary gill lamellae leading to the obliteration of interlamellar spaces was observed at 96 hours. Kidney of the treated fish responded initially with the partial loss in staining affinity, hypertrophy of epithelial cells, dilation of blood vessels supplying glomerulus and glomerulonephritis. However, the effects were more marked in Cartap treated fishes. At 48 hours of the treatment, fish exhibited signs of degeneration in epithelial lining cells and shrinkage in tubular lumina as well as glomeruli. By 72 hours, glomerular shrinkage and pycnotic changes in epithelial lining cells were enhanced in both the treatments. At 96 hours, the epithelial lining cells depicted further degenerative changes, excessive shrinkage of glomeruli and oedema. The hepatocytes of the fish exhibited hypertrophy at 24 hours of exposure in both the treatments while hyperemia was seen in Cartap treated fish. At 48 hours, dilation of blood sinusoids, partial vacuolation of hepatocytes and acentric nuclei were seen in response to Cartap treatment whereas in Carbaryl treated fishes, the hepatocytes exhibited hypertrophy, vacuolation and pyknosis. After 72 hours, the cellular boundaries of

hepatocytes were lysed at places and no demarcation was made between the individual cells in Cartap treated fishes. Also, the nuclei were pyknotic and blood sinusoids were markedly dilated. On the other hand, liver of the fish exposed to Carbaryl for the same duration showed degeneration characterized by extensive cloudy swelling of the hepatocytes to such an extent that the cytoplasm depicted "Swiss cheese like" appearance. At 96 hours, degeneration of hepatocytes, hyperplasia and disorganization of the blood sinusoids were seen in Cartap exposed fish while focal necrosis and hyperemia were observed due to Carbaryl treatment.

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Mauel, M, *et. al*. 2007, observed Tilapia are cultured worldwide and are increasing in popularity among aquaculturalists in the United States; however, data regarding normal health parameters are limited. Few hematologic and plasma biochemical values of clinically normal tilapia have been reported, but these data may be key for identifying and managing disease issues in recirculating systems. Therefore, blood was collected from clinically normal hybrid tilapia (*Oreochromis aureus* _ *Oreochromis nilotica*) housed in recirculating systems for the purpose of establishing normal hematologic and plasma biochemical reference ranges. Using standard clinical techniques the following hematologic values were determined: packed cell volume, plasma protein, leukocyte counts, leukocyte differentials, and thrombocyte counts. Additionally, the following plasma biochemical values were determined: albumin, total protein, globulins, albumin/globulin ratio, aspartate aminotransferase, alkaline phosphatase, glucose, uric acid, calcium, phosphorus, magnesium, sodium,

potassium, chloride, urea nitrogen, and creatinine. The condition of the sample was also noted (lipemic, hemolysis, and icterus). The reference ranges reported in this study can be used in the management of cultured tilapia in recirculating systems.

Agrahari, S. *et. al*. 2007, observed Monocrotophos (MCP), commonly known as azodrin, is one of the organophosphate (OP) pesticides extensively used in agricultural practices throughout the world. *Channa punctatus* were exposed to sublethal concentrations (0.96 and 1.86 mg/L) of monocrotophos for 15 and 60 days to assess the alterations in the level of some biochemical parameters in blood plasma. Significant alterations in all the biochemical parameters were found to be dose dependent. Hypoglycemia and hypocholesteremia were observed in plasma of fish at both exposure periods (15 and 60 days). Increased activities of glutamate-oxalacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), acid and alkaline phosphatase of blood plasma indicated hepatic tissue damage. Decrease in lactate dehydrogenase (LDH) content in plasma further indicated lower metabolic rate after 60 days of exposure. Significant decline in triglycerides content was observed in fish exposed to both sublethal concentrations of monocrotophos. We suggest that analysis of biochemical parameters in the fish blood may be useful in environmental biomonitoring.

Roy S. and Bhattacharya S. 2006, As3+, considered effective for aquatic weed control, has been found to be harmful to several species of freshwater teleosts. *Channa punctatus* (Bloch) exposed for 14 days to nonlethal concentrations (1/20 LC50 and 1/10 LC50) of As2O3 were sampled on days 0, 1, 2, 7, and 14. Tissue disorientation, peliosis, and vacuolization accompanied by karyolysis, apoptosis, and necrosis of hepatocytes were significant on days 1, 2, and 7. In the kidney shrinkage of the glomerulus and increase in the Bowman's space were observed on days 1, 2, and 7. Irregularities in the renal tubule including apoptotic and necrotic cells were also common. Decreased intertubular space and enlargement of the height of the brush border cells were noteworthy. Corresponding with the histopathological lesions, dose-dependent disturbances in liver and renal functions and induction of heat shock protein 70 were significant at the early phase of arsenic treatment while metallothionein was induced at a later phase of treatment.

Peebuaa P. *et. al*. 2006, observed histopathological biomarkers of toxicity in fish organs are a useful indicator of environmental pollution. Nile tilapia, *Oreochromis niloticus* exposed for one month to sediments from the Mae Klong River, Samutsongkram province, South West Thailand, which contained elevated levels of heavy metals (lead andchromium), developed abnormalities of the gills, liver and kidney. In the gill filaments, cell proliferation, lamellar cell hyperplasia, and lamellar fusion were observed. In the liver, there was vacuolatio of hepatocytesand nuclear pyknosis. Kidney lesions consisted of dilation of Bowman's space and accumulation of hyalinedroplets in the tubular epithelial cell. No recognizable changes were observed in muscle tissue. Despite the seistopathological changes, no firm correlation between levels of heavy metals in sediments and those in fish tissues could be established.

Bittencourt *et al.* 2003, investigated the reference ranges for haematological and biochemical values for Nile tilapia, *Oreochromis niloticus* (Cichlidae), cultivated in a semi-intensive system. Erythrocytes number was 6.93 ± 8.28 x 106/mm3, hemoglobin 10.52 ± 3.09g/dL of blood and hematocrit 31.85 ± 8.45%, mean corpuscular volume (MVC) 148.80 ±. 153.19µ3, mean corpuscular hemoglobin (MCH) 40.74 ± 34.19 pg and mean corpuscular hemoglobin concentration (MCHC) 35.24 ±14.92%. Total plasma protein and glucose values obtained were 3.06 ± 0.65g/dl and 60.32 ±20.22mg/dl, respectively. A correlation matrix was established to compare de degree of association among biometric data and hematological parameters, and among each of the mand biochemical values. A positive correlation was observed among weight, length, MCVand MHC, as well as between length and hematocrit. The blood glucose level waspositively correlated with weight and length, whereas total protein was positively correlated with hemoglobin. The erythrocytes count was positively correlated with hemoglobin and negatively correlated with MCV and MHC. Microscopy examination of blood smears revealed the presence of erythrocytes, neutrophils, lymphocytes, and monocytes. Under the conditions employed here, no basophils or eosinophils, nor their precursors, could be found in blood smears of Nile tilapia.

Bhuiyan A. S.*et. al*. 2001, observed *Channa punctatus* was exposed for 7 days to sumithion at a concentration of 2.0, 5.0, 5.5, 10.0, 10.5, 15.0, 15.5, 18.5, 20.0, 20.5, 25.5 and 100ppm under laboratory conditions. The fish showed severe histological changes in liver, kidney and ovary tissues. The degenerative changes included hypertrophy of cells and their nuclei, liver cord disarray, vacuolation of the cytoplasm and necrosis. In some cells the membrane was ruptured. The liver on the whole showed distance appearance. Pycnosis, vacuolation, rupture of blood vessels and hematopoietic cells of kidney fragmentation of ova were recorded in an increasing order towards the higher tested doses.

UEDA, I. K *et. al*., 2001, identified seven types of cells in the blood of *Oreochromis niloticus*: erythrocytes, thrombocytes, neutrophils, eosinophils, basophils, lymphocytes and monocytes. Glycogen was present not only in the cytoplasm of neutrophils and thrombocytes but also in some lymphocytes and monocytes. The positive reaction for myeloperoxidase and Sudan black was observed in neutrophils and eosinophils. The bromphenol blue method was strongly positive for erythrocytes and eosinophils.

Lohia S. and Srivastav, A. K. 2000, observed the infection of helminth parasites causes impact on different parameters of blood in fish. The variations in haematological values were investigated in *Channa punctatus*. Study of these blood values was divided into 2 categories: seasonal variation in related haematological values and comparative variation in some haematological values of infected and uninfected fish. Hb of male and female *C. punctatus* was lower in winter (10.0±1.22 and 9.8±0.31, respectively) and higher in rainy season (12.95±0.78 and 11.50±1.50, respectively). PCV of male and female fish was also lower in winter (32.10±1.21 and 29.70±1.35, respectively) and higher in rainy season (42.80±2.25 and 42.12±1.05, respectively). Total erythrocyte count in male and female fish was lower in winter (1.80±0.30 and 1.75±0.75, respectively) and higher in rainy season (2.85±0.55 and .98±0.26, respectively). MCV was lower in winter (178.33±16.63 and 169.71±13.9, respectively) and higher in summer (203.80±19.0 and 154.68±12.5, respectively) for male and female *C. punctatus*. Values of MCH in male fish were 55.55±3.25, 61.66±3.13, and 38.42±3.05 in winter, summer, and rainy seasons, respectively. As

for female fish, values were 56.00±3.16, 46.17±2.28, and 38.59±2.57, respectively. MCHC values of male fish were 31.15±2.57, 30.25±2.24, and 30.64±3.96 in winter, summer, and rainy seasons, respectively. Values in female fish were 32.99±2.90, 29.84±1.75, and 27.30±2.87, respectively. Hb, PCV, ESR, and total erythrocyte count of infected fish were less compared to that in uninfected fish. On the other hand, total leukocyte count was higher in infected fish than in uninfected ones. Infected *C. punctatus* had higher MCV, MCH, and MCHC than uninfected ones.

Hasan, M. R.*et. al.* 1997*,* conducted a experiment to evaluate the suitability of various oilseed cakes and *Lucaena* leaf meal as dietary protein sources for common carp fry (mean +- SE weight, 50 +- 1.6 mg). Eleven experimental diets were formulated containing different levels of mustard, sesame, linseed, copra and groundnut oil cakes and *Lucaena* leaf meal to substitute for fish meal protein up to a maximum of 75% of the total protein content. Sesame oil cake was tested at three inclusion levels (25, 50 and 75% of total protein), whereas mustard (25 and 50%), linseed (25 and 50%) and groundnut (25 and 75%) oil cakes were each tested at two levels. Copra and *Lucaena* were tested at one inclusion level (25% of total protein) only. The control diet was prepared with fish meal as the sole source of protein. All diets were isonitrogenous and contained about 40% protein. The diet containing 25% *Lucaena* meal showed the poorest performance. Apparent protein digestibilities were broadly similar (78-90%) for all diets except the diet containing 25% *Lucaena* meal which showed the poorest value (63%). With the exception of the control and 25% *Lucaena* diets, the carcass composition of experimental fish was relatively unaffected by different dietary treatments. Histopathological examination of liver revealed higher levels of intracellular lipid deposition in fish fed diet containing mustard cake. A large proportion of fish fed the 75% groundnut diet showed severe deformations of the body.

Hossain, M.A. and Shikha, F. H. 1997, observed the apparent protein digestibility (APD) of locally available feed ingredients such as *leucaena*, water hyacinth, wheat bran, rice bran and duck weed by the African catfish, *Clarias gariepinus* was evaluated at 30% inclusion level using a *fish* meal and soybean based feed as the reference diet. The results showed that the APO of reference diet was fairly high (88.62%). The APO values of other test diets ranged between 80.34 to 83.52%. The

APO in rice bran was 71.54%, followed by water hyacinth (68.52%), duck weed (66.08%), wheat bran (65.10%) and *leucaena* (60.98%). From the protein digestion coefficients obtained in this study, it is suggested that the above ingredients are suitable for inclusion in the diet of *C. gariepinus* at lower levels (lt 30%).

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Caceci, T. *et. al.* 1997, observed the stomach of *Oreochromis niloticus* was divided into three distinct regions: initial, middle and terminal, corresponding roughly to the cardiac, fundic, and pyloric portions of the mammalian stomach. Grossly, the organ showed initial and terminal portions, the former connected to the distal part of the oesophagus and the latter to the proximal portion of the intestine. There was also a middle region, forming a large blind diverticulum communicating with the first two at their point of junction. The initial or cardiac region was shorter than the middle region but longer than the terminal one, and had a smooth surface devoid of gastric pits. The epithelium in this region was simple columnar devoid of goblet cells, with glandular regions in the lamina propria. The mucosa of the middle or fundic region had gastric pits lined by columnar epithelium, and simple tubular glands filled most of the lamina propria. The terminal or pyloric part of the stomach was very short and its mucosa was slightly folded and devoid of both gastric pits and mucous glandular cells. The lining epithelium of this portion of the stomach was simple columnar and a few goblet cells were seen at its junction with the first part of the intestine. The tunica muscularis of the stomach contained skeletal muscle in the initial and terminal regions, usually intermingled with smooth muscle fibres. Skeletal muscle fibres were also observed in the first portion of the small intestine, near the junction with the stomach.

Hossain, M. A. 1996, determined proximate and amino acid composition of 15 locally available potential fish feed ingredients. The protein content of animal origin feed ingredients such as fish meal, fish silage, blood meal, silkworm pupae meal and poultry offal meal were 56.44, 56.82, 92.94, 61.06 and 75.47% respectively. The plant origin ingredients were soyabean meal, sesame meal, mustard oilcake, linseed meal, cotton seed meal, *Lucaena* meal, water hyacinth meal, duckweed, rice bran and wheat bran. Their protein content ranged between 12.62 and 45.15%. The essential amino acid lysine and methionine contents in animal protein ingredients were fairly high compared to that of plant origin. However, on the basis of amino acid profile determined, it is concluded that the above mentioned ingredients may be useful source of dietary protein for fish feed.

Osman, M. F.*et. al.1996*, carried out studied a14 week experiment to study the effects of replacing three different levels (33%, 66% and 100%) of berseem leaf meal (BLM) by *Lucaena* leaf meal (LLM) treated in four different ways (drying for 48 h at 60 degree C, autoclaved for 15 min, sprayed with 1% sodium hydroxide and incubated with rumen liquor for 24 h). Groups of Nile tilapia, *Oreochromis niloticus* (L.), fingerlings (5.07 g mean weight) were fed one of 13 isonitrogenous (30% crude protein) and isocaloric (19.67 kJ per g dry matter) diets, with two replicates (10 *fish* per aquarium) for each treatment. The results indicated that weight gain, specific growth rate, feed conversion ratio and protein utilization parameters were significantly (p lt 0.05) increased by the higher percentage of dried or cooked LLM in tilapia diets. On the other hand, the lowest growth performance and feed utilization parameters were observed in the groups fed LLM diets treated with sodium hydroxide or incubated with rumen liquor. Carcass protein and fat increased significantly (p lt 0.05) with increasing levels of LLM and simultaneously decreasing ash content.

Banerjee S, Bhattacharya S. 1994, observed histopathological changes in the head and trunk kidneys of *Channa punctatus* induced by chronic nonlethal levels of Elsan (211 ppb), mercuric chloride (16.7 ppb), and aqueous ammonia (15.64 ppm) were studied on 7, 28, 63, and 90 days of exposure. The pathology of the head kidney was characterized by degeneration and dispersion of interrenal and chromaffin tissue and necrosis in the haemopoietic elements. Kidney lesions were observed throughout the entire experimental period in fish exposed to Elsan and mercuric chloride. In contrast, the lesion induced by exposure to aqueous ammonia began to heal during the first phase of treatment. Marked abnormalities in trunk kidney histology were also found. Renal lesions consisted of minimal to mild multifocal, acute tubular epithelial degeneration, karyolysis, and dilation or shrinkage of Bowman's capsule and glomerulus. Elsan treatment resulted in a highly significant decrease in the dimension of Bowman's capsule and glomerulus at all days of sampling, except on Day 28. The response of the fish trunk kidney tissue to mercuric chloride was similar to that observed with Elsan exposure in terms of the alteration in the mean dimensions of Bowman's capsule and glomerulus. The response to ammonia was significant reduction in the size of Bowman's capsule and glomerulus throughout the experimental period except at Day 28. Little dilation of Bowman's

capsule and a significant dilation of glomerulus were found at Day 28 of ammonia exposure. This study demonstrated that a chronic nonlethal exposure to Elsan, mercuric chloride affect both endocrine and excretory parts of the kidney while ammonia specifically damages the excretory part of the kidney of *C. punctatus.*

Sethi P. and Kulkarni P. R. ,1994, studies on two varieties of *Leucaena leucocephala*, K8 and K28, whole seeds and seed fractions (kernel and hull), revealed that it is primarily the seed kernel portion of the seed that is a potential source of protein. Trypsin inhibitors, amylase inhibitors and cyanogenetic glycosides were found to be absent, while urease activity, amylase activity, saponins and haemagglutinins were present in the seeds. Reduction in urease activity on heating *L. leucocephala* seeds could be taken as an index of the reduction in mimosine content, particularly when the seeds were given high temperature treatments (e.g. autoclaving).

Dy Penaflorida, V. *et. al.1992*, observed soaking fresh *Leucaena leucocephala*(ipil-ipil) leaves in tap water (1:1, v/v; or 50 g in 500 ml) for 30-40 hours with a water change after 24 hours extracts at least 90% of its mimosine, a toxic lysine derivative. This extraction procedure is more economical and practical for fish farmers than the use of dry or moist heat or iron compounds. Soaked or unsoaked leaves of Peruvian or Hawaiian ipil-ipil formed 1/3 of trial diets fed to *Penaeus monodon* juveniles (1-2 g). After 8 weeks, the mass weight of shrimp fed the FS and soaked Hawaiian leaf diets (HLS) was significantly (alpha. = 0.05) higher than soaked (PLS) and unsoaked (PLU) Peruvian leaves. The HLS group had a significantly higher survival rate than the PLS and PLU groups but not the FS-fed shrimps survival among shrimp fed unsoaked Hawaiian leaves (HLU) was zero. It was found that Hawaiian variety of ipil-ipil leaves when soaked for 24 hours can be incorporated in the *P. monodon* juvenile diet with good survival (87%) under laboratory conditions.

Santiago, C. B. *et. al.*1988, determined the effects of dietary *Leucaena* leaf meal on reproductive performance and growth of Nile tilapia. In the preliminary trial, sexually mature Nile tilapia were fed with a control diet or a test diet which had *Leucaena* leaf meal as the only protein source for 24 weeks. Fish fed with the *Leucaena* diet lost some weight and had significantly low ($P < 0.05$) gonadosomatic index and fry production compared to those fed with the control diet. Subsequently, four

isonitrogenous diets (20% crude protein) containing varying amounts of *Leucaena* leaf meal (0, 20, 40 and 80%) were fed to Nile tilapia broodstock. Mean weight gain of ten female fish decreased as the level of *Lucaena* leaf meal in the diets increased. Females fed with the 80% *Lucaena* diet invariably lost weight. Mean weight gain of males fed with the control diet and the 20 and 40% *Leucaena* diets did not differ significantly ($P > 0.05$). However, growth of males fed with the 80% *Leucaena* diet was remarkably low. Fry production was highest for those fed with the control diet and the 20% *Leucaena* diet. Fry production decreased slightly in fish fed with the 40% *Leucaena* diet and was significantly low ($P < 0.05$) for those fed with the 80% *Leucaena* diet. The low fry production was preceded by a decrease in body weight of the female fish. However, the gonadosomatic indices of the females and the males were not markedly affected by the diets. On the basis of both fry production and growth, *Leucaena* leaf meal should not exceed 40% of the diet of Nile tilapia broodstock.

Wee, K. L. *et. al*. 1987*,* conducted an experiment to determine the suitability of treated *Leucaena leucocephala* leaf meal as an ingredient for Nile tilapia, *Oreochromis niloticus* Linn., feed. Nine experimental diets were formulated to contain 25%, 50% and 100% of the total dietary protein as plant protein using soaked (soaked in water at 30.degree. C for 48 h), sundried (sundried for 2 days) and commercial *Leucaena* leaf meal, balanced by protein from fish meal. A control diet with fish meal as the only protein source was included. All diets were isonitrogenous (30% protein) except for diets containing 100% plant protein (21% protein). The 70-day feeding trial was conducted with duplicated treatments in 2-m3 circular concrete tanks with recirculating water. There was a trend of reduced growth performance and feed utilization efficiency with increase in *Leucaena* leaf meal incorporation for all treatments. Generally, soaked leaf meal gave a significantly better growth response than sundried or commercial leaf meal. Mimosine present in the latter two treatments may have contributed to the poorer growth.

Ferraris, R. P.*et. al.1986**,*** observed the true digestibility of casein, gelatin, fish meal, defatted soybean meal and *Leucaena leucocephala* leaf meal was measured in 60- and 175-g milkfish (*C. chanos* Forsskal) in fresh- and seawater. The diets contained 45% of these feedstuffs and 1.3% of the indicator substance, chromic oxide. The

intestinal dissection method was used to collect fecal material. Results showed that the length of time between initial feeding and *fish* sacrifice did not significantly affect digestibility. Gelatin was the most digestible (90-98%) protein, regardless of size. Casein, defatted soybean meal and *fish* meal were moderately digestible (50-90%) and digestibility coefficients tended to increase as a function of *fish* size. *L. eucocephala* was the least digestible (-10-40%). The digestibility of most of these feedstuffs was less in the anterior than in the posterior intestine, and tended to be lower in seawater than in freshwater. Rate of food movement was similar in both size groups, but was significantly faster when milkfish were in seawater rather than in freshwater. The effect of salinity on digestibility may in part be due to food motility changes necessitated by alterations in osmoregulatory processes when *fish* are in seawater.

MATERIALS AND METHODS

The plant *Laucaena leucocephala* (Lam. de Wit) popularly known as Ipil Ipil or Tilikadam is a first growing tree and widely distributed in Bangladesh (kritikar and Basu 1980). The plant was taxonomically identified by Dhaka University Herbarium. The specimen is retained at the Department of Botany, University of Dhaka and its voucher specimen no. is DUH- 07.

Collection, drying and pulverization of plant material

The seed and leaf of *Leucaena leucocephala* plant was collected from Dhaka University campus during March 2008 to July 2012. The collected leaf and seed were sun-dried for seven days. After complete drying, the plant parts were pulverized in to coarse and fine powder and stored into air-tight container at room temperature.

Plate 1. *Leucaena leucocephala* (Lam. de Wit)

Apparatus and Chemical Used

Different apparatus and chemical used in the present work were supplied by the Department of Zoology and Department of Pharmacy of Dhaka University and some were also purchased personally. Below are the name of those apparatus and chemicals.

Apparatus used

1. Conical flask (100ml, 250ml, 500 ml)
2. Beaker (50ml, 100 ml, 250ml)
3. Reagent bottle (50ml, 100 ml, 250ml)
4. Piipette (10ml, 20 ml, 30ml)
5. Measuring Cylinder (100 ml, 250ml)
6. Centimeter scale
7. Thermometer
8. P^H meter (Model 7-1, New York, USA)
9. Glass aquarium
10. Funnel (large, small)
11. Net and rope
12. Fine muslin cloth
13. Filter paper
14. Silica gel (PF254) pre-coated (0.2 mm) aluminium foil
15. Glass vial (5 ml, 10ml)
16. Glass tube
17. Spotting capillaries for applying samples
18. Ultra-violet lamp
19. Burette
20. TLC developing tank, a glass chamber
21. Rota evaporator
22. Dry oven
23. Single pan balance cert -0 g, 3119 g capacity- USA, No.J44631
24. HACH's FF-2 Aquaculture Test Kit (Cat. No. 2430-01)
25. Bleder (Moulinex- France , 242)
26. Syringe (2 ml, 3 ml, 6 ml)
27. Automatic Blood Cell Counter Mythic (France)
28. Centrifuge machine Gallenkamp (U.K.).
29. Automatic Analyzer, Dimension RX L Max DADE Behring Siemens (U. S. A.).

30. Digital 9key. Erma Inc.
31. Automatic tissue processor
32. Paraffin dispenser
33. Rotary microtome
34. Warmer / Stressor.
35. Dissecting tray
36. Dissecting needles
37. Scissors
38. Forceps
39. Scalpel
40. Petredishes
41. Hand lens
42. Dropper
43. Brush
44. Watch glass
45. Microtome machine
46. Knife
47. Slides & cover slip
48. Anti cutter
49. Tape
50. Marker
51. Hand globes

Chemical used

1. Absolute ethyl alcohol (Merck, Germany)
2. Ethyl alcohol (50%, 70%, 80%, 85%, 90%, 95% 100%)
3. Hexane
4. Ethylacetate
5. Methanol (Merck, Germany)
6. Xylene
7. Acetic acid
8. Chloroform
9. Distilled water
10. Dragendroff reagent
11. Vanillin-H_2SO_4 acid spray reagent
12. Ethylene Diamine Tetra Acetic Acid
13. Giemsa stain

14. 1% P^H 64 Phospate
15. Bouin's fixative
16. Formalin (40%formaldehyde)
17. Paraffin
18. Haematoxyline
19. Eosin
20. Ammonia
21. DPX
22. Saline water

3.1 Chemical Investigation

3.1.1 Extraction with Ethanol

The pulverized coarse powder of the dried plant parts were successively extracted with absolute ethanol. Coarse powder of the plant parts were extracted with ethanol by means of cold extraction for 7 consecutive days, after which the extracts were collected and filtered by cotton plug followed by vacuum filtration to remove the remaining dust particles.The ethanolic extracts were then concentrated to dryness in a rotary evaporator at 45°C under reduced pressure. Finally the ethanol extracts were obtained after freeze drying (table-1).

Table 1. Extraction of leaf and seed by ethanol

Sample	Coarse powder kg / Litre	Extract obtained g	% of yield
Seed	1 kg/ 3 L.	10.5	1.1%
Leaf	1 kg/ 3 L.	11.5	1.15%

3.1.2 Phytochemical investigation of plant parts

Thin Layer Chromatographic analysis of ethanolic extract of leaf and seed of *Leucaena leucocephala..*

3.1.3 Thin Layer Chromatography (TLC)

The Ethanolic extract of seed and leaf of *Leucaena leucocephal* was first analyzed by thin layer chromatography using silica gel (PF254) pre-coated (0.2 mm) aluminium foil as solid phase and different solvent systems as mobile phase. Prior to application of extract, the pre-coated silica plate was activated by heating for 70 minutes at 110°C.

Different solvent systems used for TLC of ethanolic extract of leaf and seed of *Leucaena leucocephala .*

Solvent system	**Proportion**
n-Hexane : Ethylacetate	90:10
n-Hexane :Ethylacetate	50:50
n-Hexane : Ethylacetate	0 : 100
Ethylacetate : Methanol :Acetic acid	100:2drop:2drop

3.1.4 Application of Sample

A small amount of ethanolic-extract was dissolved in *n*-hexane to get approximately 1% solution. A small amount of this sample was applied on the activated silica gel plate in the form of a band at the edge of the plant using a spotter. Afterwards applied sample was completely dried before developing the chromatogram.

3.1.5 Chamber saturation

A selected solvent was poured into a TLC tank and a filter paper was placed inside the tank in a conventional way. The tank was made air-tight for few minutes to saturate the inside space with the solvent vapor.

3.1.6 Development of Chromatography

For ascending development of the thin layer chromatogram the spotted plate was placed in the TLC tank. The spotted end of the plate was placed on the flat surface of the tank in such a way that the sample spot remained above the surface of the solvent. The plate was then left for development of the chromatogram. When the solvent front reached 1cm below the upper edge of the plate, it was taken out of the tank and dried. The dried plate was then identified for the presence of compounds.

3.1.7 Detection of Compounds

The presence of compounds, if any, in the chromatogram was detected by examination of the chromatogram:

(1) Ultra-violet light (λ_{254} nm and λ_{366} nm): The chromatogram was examined under UV light to detect florescent compounds exhibit glowing spots as well as pigment type compounds appearing as red spots. The distinct spots were marked.

(2) Vanillin-H_2SO_4 acid spray reagent: The developed plate was sprayed within Vanillin-H_2SO_4 and heated at 110^0 C for 3 – 5 minutes.

(3) Dragendroff reagent: The developed plate was sprayed within Dragendroff reagent and heated at 110^0 C for 3 – 5 minutes.

3.1.8 Determination of R_f Value:

R_f value is a characteristic feature of a compound in a specific solvent system. It helps in the identification of compounds. R_f value can be calculated by the following formula:

$$R_f \text{Value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

3.2 Biological Investigation

The experimental fishes were *Channa punctatus* (Bloch &Schneider, 1801), family Channidae and *Oreochromis niloticus* (Linnaeus, 1758), family Cichlidae.

3.2.1 Collection of fishes:

During the present experiment 300 individuals of *Channa punctatus* and 300 *Oreochromis niloticus* were investigated. These fishes were collected from the ponds located at Village Panchrukhi, Upazila Araihazar, District Narayanganj from the month of April 2008 to September 2012.

Identification of Fishes:

Channa punctatus(Bloch & Schneider 1801)and *Oreochromis niloticus* (Linnaeus, 1758), the fishes were identified according to the description of Day (1978), Munro (1955) and Shafi and Quddas (1982) before using for bio-assays.

Maintenance of fishes:

The fishes were maintained and stocked in ten glass aquariums. Tap water was supplied in the aquaria. Necessary arrangements were taken for aerating the water regularly so that the fishes might not suffer from oxygen depletion. The fishes were acclimatized for a week before they were used for the experiment. During acclimatization small fishes, dried fishes and rice-bran were given as food. The average standard length and weight of the fishes were noted. The water quality was recorded by HACH's FF-2 Aquaculture Test Kit (Cat. No. 2430-01) during the experiment. The fishes were observed carefully for any disease or abnormality. At last the healthy disease free fishes were taken for bio-assays.

Selection of fish food:

During the period of acclimatization small fishes eg. mola, tengra, puti, kachki, prawn were given as fish food. Each day one kind of food was given. In this observation it was found that the fishes liked to eat small fishes, specially kachki and molafishes.

The experimental specimens that were used in the bioassays were *Channa punctatus* and *Oreochromis niloticus*. The average total length, standard length and weight of the fishes used in the bio-assay were as follows:

Table 2. Measurement of various parameters of fishes under investigation

Name of the fish	Total length (cm)	Standard length (cm)	Weight (g)
Channa punctatus	17.5 ± 3.5	14.5 ± 3.5	74.5 ± 10.5
Oreochromis niloticus	16 .5± 2.5	13.5 ± 2.5	75.5 ± 15.5

Preparation of the sample food

For the preparation of the experimental food following ingredients were used *Leucaena leucocephala* (Ipil Ipil) leaf and seed powder, small fishes, kachki, mola and flour.

Equipments

Blender (Moulinex- France , 242), Balance, Petredish

Selection of Food Amount

Leucaena leucocephala leaves and seed powder were used as fish food in the present study. In Bangladesh *L. leucocephala* leaf used as fish food. The amount which had been used in the bioassays were calculated in g/kg. body weight for leaf and seed powder. Total consumption of food was determined on the trial basis which is about 2% of total body weight of each fish. The experimental fishes were divided into several groups according to different amount of food. The total body weight of each group of fish was taken separately. The food was given 2% of the total body weight of each group. The experimental food *L. leucocephala* was given mixed with the food after a certain percentage of the total body weight of each group.

Preparation of the sample food of *Leucaena leucocephala* leaf

The food was prepared according to the total body weight of the each group separately. Small fishes and *L. leucocephala* leaf powder were weighted according to the selected groups. Small fishes (kachki and mola) were crushed by the blender and kept in the marked petredishes for mixing the leaf powder for respective group. Different amount of leaf powder were mixed into the crushed fishes and a small amount of flour was mixed binding the food ingredients. The mixed ingredients were then rounded and prepared several small balls to feed the fishes (table 3, 4).

Table 3. *Channa punctatus* fed on *Leucaena leucocephala* leaf

Study group	Number of fish	Total weight of fishes g	Food supplied in (g)% of total body weight	leaf added in % of total food	Amount /Kg body weight g
1	10	757	2%	0%	0
2	10	676	2%	25%	3.38
3	10	723	2%	30%	4.34
4	10	729	2%	35%	5.10
5	10	776	2%	40%	6.21

Table 4. *Oreochromis niloticus* fed on *Leucaena leucocephala* leaf

Study group	Number of fish	Total weight of fishes g	Food supplied in (g)% of total body weight	leaf added in % of total food	Amount /Kg body weight g
1	10	939	2%	0%	0
2	10	754	2%	25%	3.77
3	10	793	2%	30%	4.76
4	10	762	2%	35%	5.33
5	10	1053	2%	40%	8.42

Preparation of the sample food of *Leucaena leucocephala* seed

The food was prepared according to the total body weight of the each group separately. Small fishes and *Leucaena leucocephala* leaves powders were weighted according to the selected groups. Small fishes (kachki and mola) were crushed by the blender and kept in the marked petredishes for mixing the doses of seed powder for respective group. Different doses of seed powder were mixed into the crushed fishes and a small amount of flour was mixed binding the food ingredients. The mixed ingredients were then rounded and prepared several small balls to feed the fishes (table 5, 6).

Experimental fishes fed *L. leucocephala* seed

Plate 2. (a) Control group, (b) Experimental group, (c) Food ingredients, (d) Feeding of *Leucaena leucocephala* seed food.

Table 5. *Channa punctatus* fed on *Leucaena leucocephala* seed

Study group	Number of fish	Total weight of fishes (g)	Food supplied in (g)% of total body weight	Seed added in % of total food	Amount / kg bodyweight (g)
1	10	757	2%	0%	0
2	10	597	2%	20%	2.38
3	10	636	2%	25%	3.18
4	10	614	2%	30%	3.68
5	10	663	2%	35%	4.64

Table 6. *Oreochromis niloticus* fed on *Leucaena leucocephala* seed

Study group	Number of fish	Total weight of fishes (g)	Food supplied in (g)% of total body weight	Seed added in % of total food	Amount / kg bodyweight (g)
1	10	713	2%	0%	0
2	10	696	2%	20%	2.78
3	10	670	2%	25%	3.35
4	10	690	2%	30%	4.14
5	10	704	2%	35%	4.93

The experiment was continued for two months. Each of the experiment was replicated thrice.

3.2.2 Collection and preparation of blood

Many techniques have been devised in the past for collection of blood samples from various sites of fishes (Lied *et. al.* 1975; Blaxhall and Daisley, 1973 and Hesser, 1960). The cardiac puncture, venous puncture and severance of the caudal peduncle have been commonly used. During the present investigation, blood was collected from cardiac puncture. After collecting the blood by disposable syringe (3ml and 6ml) desired amount of blood was delivered into a vial containing EDTA (Ethylene Diamine Tetra Acetic Acid). The blood was mixed gently with the anticoagulant. A portion of the collected blood was used in haematological determination. The remaining blood was transferred to another tube to determine the biochemical parameters.

Collection of Blood from the experimental fishes

Plate 3. Collection of blood from *Channa punctatus*

Plate 4. Collection of blood from *Oreochromis niloticus*

3.2.3 Preparation of blood films

Smears of the blood sample obtained by cardiac puncture are prepared by placing a drop of the sample on one edge of an alcohol–cleaned slide and distributing it the length of the slide by another slide held at 45^0 to first. The smear is allowed to air dry, immersed in absolute methanol for at least 5 minutes and again air dried before being stored or stained.

Staining Techique

- At first Giemsa stain in 1% P^H 64 Phospate buffer used to stain the smeared slide.
- Then washed with distilled water to remove the extra color and dried for 5 to 10 minutes.
- After this deep in weight stain for 2 to 3 times and again washed with distilled water and dried in air.

3.2.4 Total Erythrocyte (RBC) and Leucocyte (WBC) counts

Haematological parameters Erythrocyte (RBC), Leucocyte (WBC), Haemoglobin (Hb), Hematocrit (Hct), were determined by rolling the blood sample in an automatic Cell Counter Mythic (France).

Preparation of blood for counting

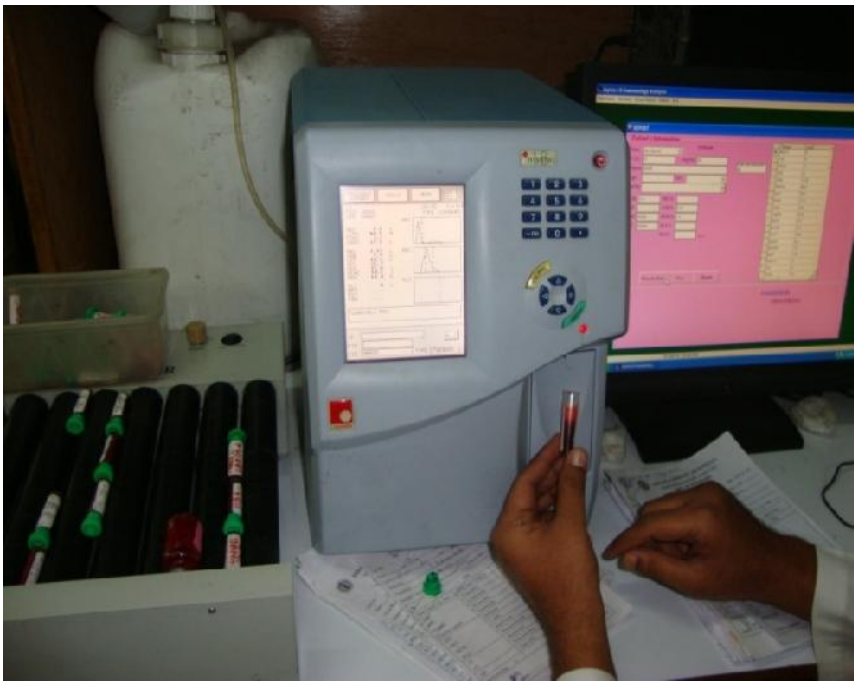


Plate 5. (a) Blood sample,

(b) Automatic Cell Counter, Mythic (France)

3.2.5 Differential counts of Leucocyte (WBC)

For the differential count of the WBC blood smears were made and stained following Klontz (1972), and counting was done with the help of a leucocytometer (Digital 9key. Erma Inc.). Identifications were done following Mahajan and Dheer (1979); Ellis (1977); Williams & Warner (1976) and Pillay (1958).

MCV Determination

The Mean Corpuscular Volume (MCV) can be determined from the data taken. To calculate the MCV, expressed in femtoliters (fl, or 10^{-15}L), the following formula is used:

$$\text{MCV} = \frac{\text{Hematocrit (\%)} \times 10}{\text{RBC count (millions/mm}^3\text{ blood)}}$$

MCH Determination

The Mean Corpuscular Hemoglobin (MCH) can be determined from the data taken. The equation is as follows :

$$\text{MCH} = \frac{\text{Hemoglobin (g/100ml)} \times 10}{\text{RBC count (millions/mm}^3\text{ blood)}}$$

MCHC Determination

The Mean Corpuscular Hemoglobin Concentration (MCHC) .To calculate the MCHC, expressed as grams of hemoglobin per 100 ml packed cells, the following formula is used:

$$\text{MCHC} = \frac{\text{Hemoglobin (g/100ml)} \times 100}{\text{hematocrit (\%)}}$$

Biochamical Determination

Fish from each experimental group and control group were bled from the cardiac puncture into sterilized glass vials containing the anticoagulant 1% ethylene diamine tetra acetate (EDTA). The sample that kept for biochemical determination centrifuge by centrifuge machine Gallen kamp (U.K.). The blood was centrifuged for 15 min at 400 rpm. Then only the blood serum was taken for biochemical determination. Biochemical parameters, total protein, albumin, glucose, urea, creatinine and cholesterol were determined by analyzing the blood sample in an Automatic Analyzer, Dimension RX L Max DADE Behring Siemens (U. S. A.).

3.2.6 Statistical Analysis

All data were presented as mean ± standard deviation. The haemamatological and biochemical parameters of blood were compared between each experimental group and control group by Pearson correlation coefficient and graphs were drawn. The means of haematological and biochemical variables were compared among the experimental food groups were showed by analysis of variance (ANOVA) followed by Tukey Honest Significant Difference (HSD) Post hoc for multiple comparisons. Statistical software SPSS version 20 was used to analyze the data. The level of significance was set at 0.05.

Biochemical Test

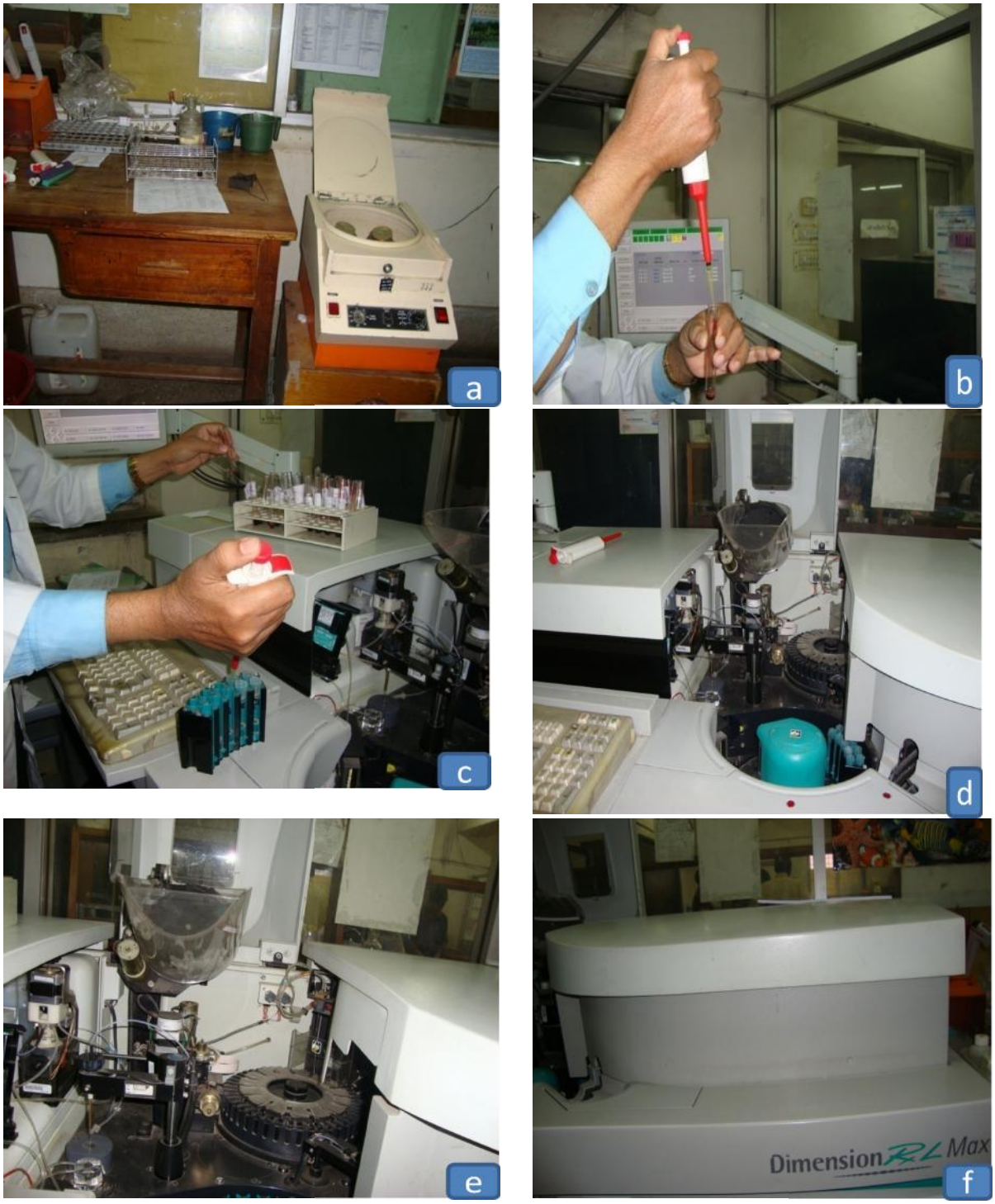


Plate-6 . (a) Centrifuge machine, (b) Serum was collected with pipette, (c) Test tubes filled with serums, (d) Tubes were placed in the Automatic Analyzer, (e) and (f) Automatic Analyzer, Dimension RX L Max DADE Behring Siemens (U. S. A.)

Dissection of *Oreochromis niloticus*

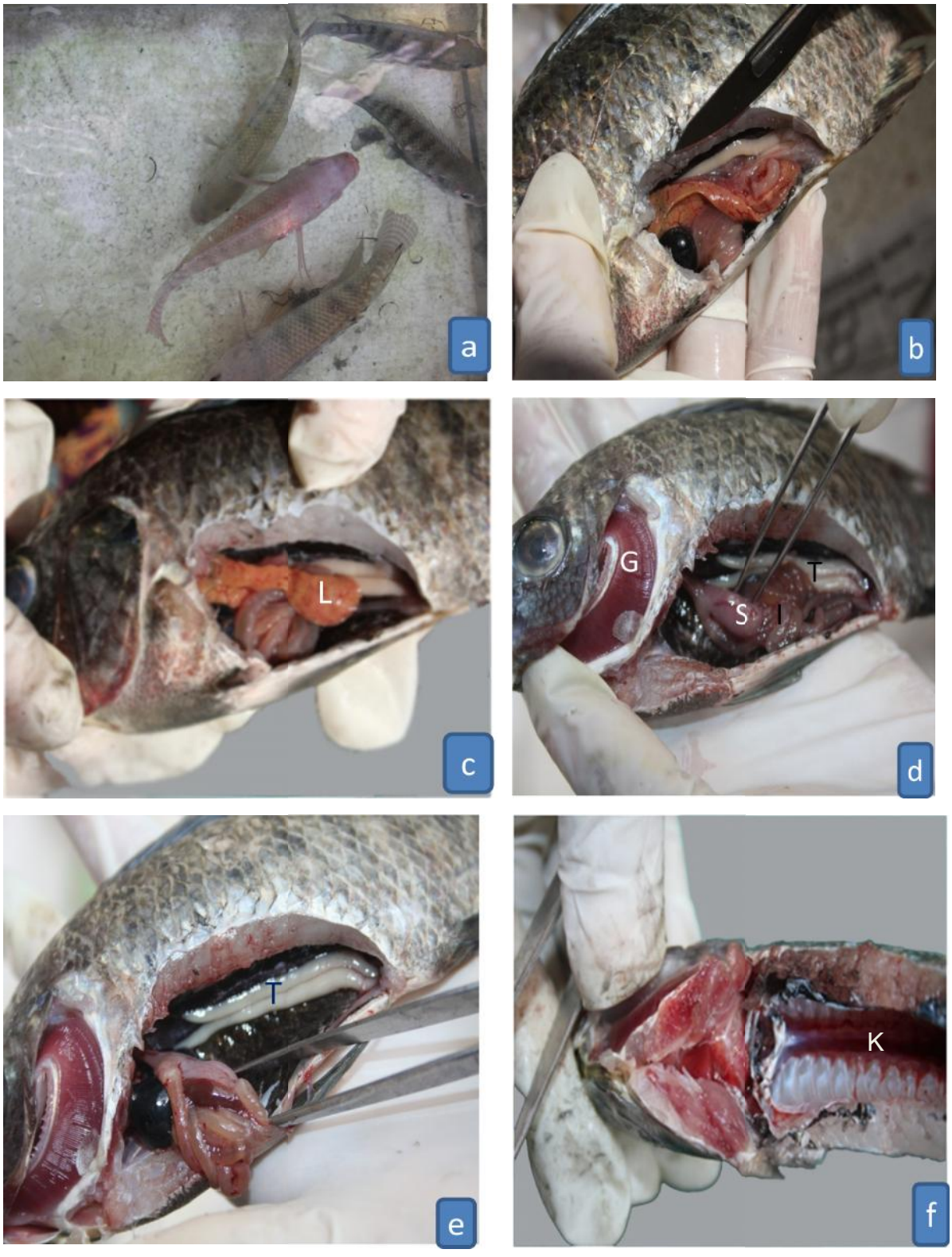


Plate-7. (a) *Oreochromis niloticus,* (b) Dissection of *O. niloticus,* (c) L-Liver, (d) G- Gill, S- Stomach (e) I- Intestine, T-Testes (f) K- Kidney

3.3 Histopathological Study

Histopathological investigation is generally carried out on thin sections of cell-culture material, organs or whole small fish (up to 5mm thick). The fixed blocks of tissue were processed and embedded in paraffin wax, and 4-5μ m thick sections were prepared. The sections were stained with haematoxylin and counter stained with eosin.

3.3.1 Preparation of permanent histopathological slides: The following apparatus and reagents were used to prepare histopathological slides.

Apparatus:

1) Automatic tissue processor.
2) Paraffin dispenser.
3) Rotary microtome and
4) Warmer / Stressor.

Reagents :

1. Bouin's fixative:
   a) Picric acid, saturated aqueous solution –75ml
   b) Formalin (40%formaldehyde)– 25ml
   c) Glacial Acetic acid – 5ml
2. Wash with 70% alcohol
3. Alcohol (50%, 70%, 80%, 85%, 90%, 95% 100%)
4. Paraffin
5. Haematoxyline.
6. Eosin.
7. DPX and
8. Ammonia

3.3.2 Fixation of tissue sample

After dissection, the collected tissue were quickly washed with physiological saline solution (0.75%) and then fixed in Bouin's fluid.

Next wash with 70% alcohol (ethanol).

3.3.4 Processing of tissue

a) Dehydration:

The fixed tissues were gradually dehydrated in ascending concentration of alcohol from 50% - 100% by automatic tissue processor at Bangabandhu Sheik Mujib Medical University Pathology laboratory.

b) Clearing :

After dehydration the tissue were cleared in xylene by automatic tissue processor.

c) Paraffin impregnation :

A paraffin bath with temperature range of 60^0C – 70^oC was used .The melting point of the paraffin was 58^0 C – 62 ^{0}C The tissue were treated in molten paraffin for 1 – 2 hours.

d) Paraffin embedding:

Embedding in melted wax for 30 – 45 minutes. The tissue was boiled with paraffin wax in incubator at 60 ^{o}c. In paraffin the tissue become roasted.

e) Block making:

Paraffin dispenser was used for block making. Molten paraffin was then poured into a block of the iron blocked plate. The tissues were carefully embedded in a proper plan. The blocked plate was allowed to harden in the ice chamber of a refrigerator.

f) Trimming:

The blocks were then trimmed properly to the size of the tissue by cutting off the extra paraffin and were kept in the ice chamber for sometime before cutting the section.

g) Section cutting:

The block was fitted to the microtome for sectioning. A sharpened microtome knife was used for cutting sections. The temperature of the water bath was maintained between 45^0c- 50^0c for stretching the ribbon on it. Sections were cut at 4-5 micron in thickness.

h) Affixing:

The ribbon of tissue was attached on the slide by means of Mayer's albumen.

Mayer's albumen: Fresh egg's white – 50ml
Glycerin - 50ml
Sodium salicylate - 5ml

In 20 cc distilled water 3-5 droop of Mayer's albumen was shacked and used.

i) Mayer's albumen was put in the slide. The ribbon of the tissue was arranged in rows putting the slide. The slides were then kept in slanting position for sometimes to drain out the water and then allowed to dry at 25^0c-34^0c in warmer.

j) Deparafinization:

The slides with ribbons containing tissue were kept in the warmer for melting at 60^0 C for ten minutes. This was followed by immersing the slides in xylene for ten minutes to remove the wax

k) Rehydration of tissues:

The slides with sections of tissues were gradually rehydrated in descending concentration of ethyl alcohol from absolute alcohol to 70%- keeping ten minutes in each. At the end of this process, tap water was used to remove alcohol.

l) Staining :

For staining the following procedure were used.

1. Dipped in Haematoxyline – 5 minutes and washed properly with running water.
2. Dipped in 0.5% alcohol – 15 second, washed again with running water.
3. Dipped in Ammonia – 1 minute washed with running water.
4. Dipped in Eosin – 01 to 02 minutes.

m) Dehydration:

The slides were then dehydrated gradually with alcohol in the following ways:

Alcohol – 70% for 5 minutes
Alcohol – 90% for 3-5 minutes
Alcohol – 100% for 15 minutes.

n) Cleaning:

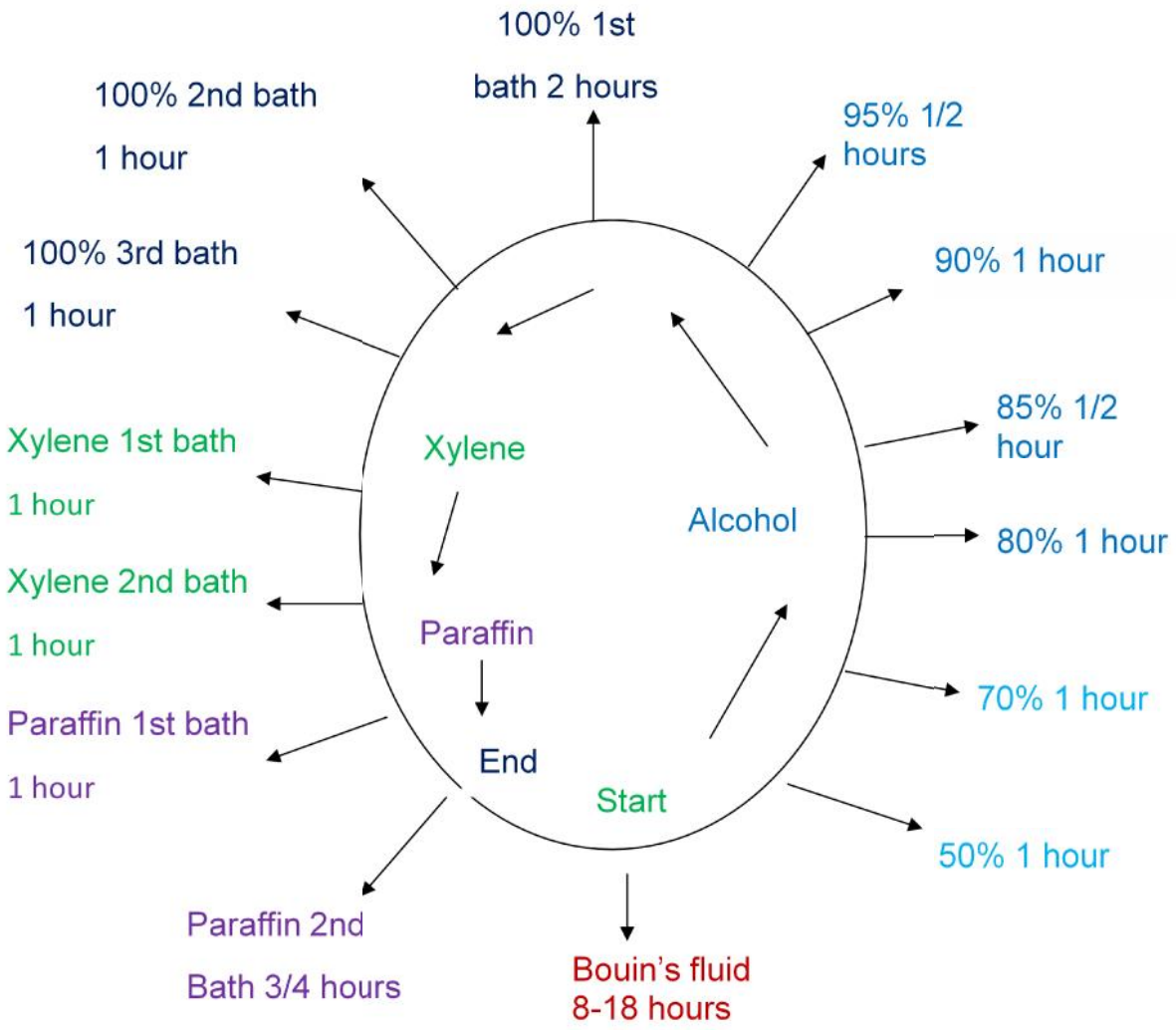
The stained tissues were cleaned in xylene-I, Xylene-II and Xylene-III for two minutes each.

o) Mounting:

After cleaning permanent mounting of the slide were made by paramount.

p) Labeling

Finally the slides were labeled according to samples.



AUTOMATIC TISSUE PROCESSOR