EFFECT OF *MANGIFERA INDICA* L. *(*MANGO) KERNEL ON *CLARIAS GARIEPINUS (*AFRICAN CATFISH) FINGERLINGS INFECTED WITH *AEROMONAS CAVIAE*

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MAY, 2014

DECLARATION

I declare that the work in this thesis entitled ‘‘**Effect of *Mangifera indica* L. (Mango) Kernel on *Clarias gariepinus* (African catfish*)* Fingerlings Infected with *Aeromonas caviae***’’ has been carried out by me in the Department of Veterinary Pharmacology and Toxicology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree or diploma at this or any institution.

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CERTIFICATION

This thesis entitled **''EFFECT OF *MANGIFERA INDICA* L. (MANGO) KERNEL ON AFRICAN CATFISH (*CLARIAS GARIEPINUS)* FINGERLINGS INFECTED WITH *AEROMONAS CAVIAE*''** by Muhammad Talba AHMAD meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

A number of approaches were employed to control diseases in fish with particular emphasis on use of chemotherapeutic agents. However, application of antibiotics in aquaculture is expensive and leads to antibiotics residue in fish, which could cause the development of antibiotic resistance in human. In this study, phytochemical analysis was done to detect the presence of secondary metabolites in the *M. indica* kernel. The *in vitro* and *in vivo* antimicrobial effects of aqueous and methanol extracts of mango kernel on *A. caviae* using agar well diffusion method were evaluated. In the *in vivo* trial, two hundred and fourty fingerlings were randomly divided into four groups of sixty. Fish in group A, B, C and D were fed diet containing 0, 1, 5 and 10 g/kg of mango kernel dry fish diet, respectively for 60 days. Some haematological parameters of fish were examined at 20 and 40 days of feeding the diet. Twelve fish from each group were challenged with *A. caviae* 60 days post feeding and clinical signs, mortalities and gross lesions were observed and recorded over 14 days post-infection. Phytochemical analysis of mango kernel revealed the presence of triterpenes, tannins, glycosides, saponins and flavonoids. The result obtained following the limit dose test demonstrates that the lethal dose (LD_{50}) of *M. indica* kernel powder is greater than 60 g/kg of feed. The methanol extract when used at concentrations that ranges from 50 to 500 mg/ml inhibited growth of the bacteria, with zone of inhibition ranging between 16 ± 2.41 to 24 ± 0.58. The antibacterial activity of the methanol extract was not significantly different from the standard antibacterial agents used in the study (gentamicin, enrofloxacin, neomycin, chloramphenicol and erythromycin). However, *M. indica* powdered kernel solution did show antibacterial activity against *A. caviae* at lower concentrations (166, 125, 100, 55.6 and 50 mg/ml) with smaller zones of inhibition that

ranges from 8 ± 1.00 to 11 ± 1.00, with no measurable zones of inhibition at higher concentrations of 250 and 500 mg/ml. The results demonstrated that the serum of fish treated with mango kernel at different concentrations produced no antibacterial effect against *A. caviae.* However, the total leukocyte counts were significantly higher in fish treated with mango kernel at 10 g/kg of feed 40 days post-treatment. Less survivability was observed in fish that were not treated with diet containing mango kernel (50% survivability) up to day 14 after infection. The groups fed 5 and 10 g/kg mango kernel dry diet showed highest percentage survival (100%). Results of the present study clearly demonstrated that only methanol extract of mango kernel showed good antibacterial activity. The present study also confirmed the efficiency of the organic solvent for extraction of plant constituent compared to water. The study indicates that mango kernel protects *Clarias gariepinus* against *A. caviae* infection by enhancing the survivability of the treated fish.

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ABBREVIATION

A.D	Anno Domini
B.C	Before Christ
ß	Beta
cm	Centimeter
CBN	Central Bank of Nigeria
CDC	Centers for Disease Control
CIN	Cefsulodin-irgasan-novobiocin
°C	Degree Celcius
DC	Deoxycholate citrate agar
DNA	Deoxyribonucliec acid
EA	Ellagic tannins
FAO	Food and Agriculture Organisation
Ft	Feet
GA	Gallotannins
GESAMP	Group of Experts on the Scientific Aspect of Marine EnvironmentalProtection
GSI	Gonadal Somatic Index
Hb	Hemoglobin
HE	Hekteon agar
HG	Hybridization group
HPLC	High Performance Liquid Chromatography
Kg	Kilogramme
kD	Kilodalton
lb	Pond

LC_{50}	Lethal Concentration Fifthy
LD_{50}	Lethal Dose Fifthy
LPS	Lipopolysaccharide
m	Meter
M ± SEM	Mean ± Standard Error of Mean
M ± SD	Mean ± Standard Deviation
Mg	Miligramme
Min	Minutes
ml	Mililiter
mm	Milimeter
MRSA	Methacillin Resistant Staphyloccus Aureus
NCCLS	National Committee on Clinical Laboratory Sciences
NMR	Nuclear Magnetic Resonance Spectroscopy
OECD	Organisation for Economic Cooperation and Development
PCV	Parked Cell Volume
RBC	Red Blood Cells
rRNA	Ribosomal Ribonucleic Acid
SS	Salmonella-Shigella Agar
TCBS	Thiosulfate citrate-bile salt sucrose
TLC	Total Leucocyte count
TP	Total protein
UK	United Kingdom
US	United States
US$	United States Dollars
XLD	Xylose lysine deoxycholate sugar
µl	Microlitre

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Aquaculture is one of the fastest growing food-producing sectors around the world (Harikrishnan *et at.,* 2011). The world's total production of fish and shellfish (including mollusk and crustacea) was 99 metric tonne in 1990 and it increased to 122 metric tonne in 1997 (Hill, 2010). According to Food and Agriculture Organization (FAO), the global aquaculture production has increased from about 28.3 million tonnes to 40 metric tonnes in 2009 (FAO, 2009). According to Harikrishnan *et al*. (2011), among various kinds of cultivated organisms, many marine and freshwater finfish and shellfish species constitute an important industry with their production increasing every year. Aquaculture fish production increased significantly over the past few decades necessitating intensive fish culture practices.

In aquaculture, disease control using chemotherapeutic agents has been complicated by the misleading advice provided to the farmers by feed and chemical companies regarding the use of antibiotics and other therapeutic agents (FAO, 2003a). In the intensive aquaculture system, application of antibiotics and chemotherapeutics as prophylactic measures has been widely criticized for their negative impacts like immunosuppression, residue accumulation in tissues (Rijkers *et al*., 1980; FAO, 2003b; Harikrishnan *et al*., 2009a, 2009b) and the development of drug resistant pathogens and environmental pollution (Smith *et al*., 1994). International agencies recommends that the use of antibiotics should be restricted to therapeutic purposes only, and that preventive approaches should be preferred in fish disease management over costly post disease treatments (GESAMP, 1997; FAO, 2005).

Commercial vaccines are expensive for fish farmers and are specific against particular pathogens (Raa *et al*., 1992). It is widely demonstrated that farmed fish are more susceptible to diseases cause by infectious agents due to the stress caused by intensive rearing (FAO, 2003a).

1.1.1 *Aeromonas caviae*

Aeromonas caviae is a ubiquitous bacteria in aquatic environments (Janda and Abbott, 2010).It is known to cause several diseases including,Motile Aeromonas Septicemia (MAS) in carp, tilapia, perch, catfish, and salmon, red sore disease in bass and carp, and ulcerative infections in catfish, cod, carp, and goby (Joseph and Carnahan, 1994).

1.1.2 Mango (*Mangifera indica*)

Mangifera indica L., which belongs to the *Anacardiaceae* family, is a large evergreen tree of tropical and subtropical regions. *Mangifera indica* grows in the tropical and subtropical region and its parts are commonly used in folk medicine for a wide variety of remedies (Coe and Anderson, 1996). Different parts of mango have a broad range of medicinal properties, such as antimicrobial (Kabuki *et al*., 2000; Keita *et al*., 2004), antiviral, antifungal (Cojocaru *et al*., 1986), anti-inflammatory (Garrido *et al*., 2004), anti-diarrhoeal (Sairam *et al*., 2003), antioxidant activity (Scartezzini and Speroni, 2002; Anila and Vijayalakshmi, 2003), antitumor (Garrido *et al*., 2004), as well as immunomodulatory (Makare *et al*., 2001). Of all the by-products of mango, mango kernel is the cheapest and readily available. Huge amounts of mango kernel, which is not presently utilized can be utilized as feed to enhance the non-specific immunity in fish (Sahu *et al*., 2007). The mango kernel is also used as a non-conventional feed source for growth and feed utilization

of *Oreochromis niloticus* fingerlings (Omoregie *et al*., 1991), broiler chicken (Joseph and Abolaji, 1997) and rat (Okai and Aboagye, 1990).

1.2 Statement of Research Problem

According to Harikrishnan *et al*. (2011), in recent years due to intensive aquaculture farming practices, infectious diseases pose a major problem in the aquaculture industry, causing heavy loss to the fish farmers. A number of approaches were employed to control diseases including sanitary prophylaxis, disinfection, and chemotherapy with particular emphasis on the use of antibiotics. However, the application of antibiotics and chemicals in aquaculture is often expensive and undesirable since it leads to antibiotic resistance and consumer reluctance to buy fish with antibiotics residue.

Chemotherapy is widely applied to control or prevent occurrence of infectious diseases. Several antibiotics have been used to control finfish and shellfish diseases. The antimicrobials used include amoxicillin, enrofloxacin, erythromycin, furazolidone and oxytetracycline (Smith *et al.,* 1994; Agnew and Barnes, 2007). However, application of chemotherapeutic agents could affect fish leading to mortality and other detrimental side effects (Chong and Chao, 1969). The use of formalin in fishes results in severe gill damage and repeated treatments with nitrofurazone causes ulcerative dermatitis (Punitha *et al.,* 2008). Furthermore, the use of disinfectants and antimicrobial drugs had limited success in the prevention or cure of aquatic diseases (Sakai *et al*., 1991; Gulliver *et al*., 1999; Sakai, 1999; Michael, 2001; Salisbury *et al*., 2002). The pathogens may also transfer their antibiotic-resistance genes into human pathogenic bacteria thus posing a threat to human health and environmental problems (Smith *et al*., 1994; Alderman and Hastings, 1998;

MacMillan, 2001; Abutbul *et al*., 2004; Cabello, 2006), leading to failure of antibiotic treatment in some life-threatening conditions which limits the use of traditional therapeutic substances (Miranda and Zimelman, 2001; Radu *et al*., 2003).

1.3 Justification

The estimated economic loss due to diseases in aquaculture is more than US$ 400 million in China in 1993, US$ 17.6 million in India in 1994, and over US$ 500 million in Thailand in 1996 despite the partially successful preventive measures including sanitary prophylaxis, disinfection, antibiotics, vaccines and chemotherapy in the last 20 years (Hill, 2010). In countries affected by fish diseases like EUS (Botswana, Namibia and Zambia), diseases negatively impacted on the livelihood and food fish source of the communities dependent on subsistence farming (Bondad-Reantaso *et al*., 2012). Fish production is a major component of the agricultural sector and a vital source of protein to majority of Nigerians. According to Adekoya and Miller (2004), fish and fish products constitute more than 60% of the total protein intake in adults especially in rural areas. There is a complimentary large appetite for fish in Nigeria with an estimated demand of about 1.5 million metric tons of fish in 2006 (Davies *et al*., 2008), which has grown to 2.66 million in 2010 (CBN, 2010b), these figures places Nigeria among the largest fish consumers in the world.

Herbal medicines are also known to exhibit anti-microbial activity, facilitate growth, and maturation of cultured species; besides under intensive farming the anti-stress characteristics of herbs will be of immense benefit without posing any environmental hazard (Harikshnan *et. al.,* 2003). Administration of herbal extracts or their products at various concentrations through oral (diet) or injection route enhance the innate and adaptive

immune response of different freshwater fish, marine fish and shellfish against bacterial, viral, and parasitic diseases (Harikrishnan *et al*., 2011).

The accumulation of chemicals in the environment and in the fish have led to the imposition of stringent regulations that limit the use of antibiotics and a number of chemicals (Alderman and Hastings, 1998; Treves-Braun, 2000) that are harmful to the environment as well as consumers (Smith *et al*., 1994). The massive use of synthetic antimicrobials for disease control has been suppressing growth in aquatic animals. Indeed food producing animals are one of several potential sources of antibiotic resistant bacteria which may spread from animals to man via the food chain (Harikrishnan *et al*., 2011). Recently, a growing interest has emerged in using herbs in animal feeds by both researchers and feed companies (Harikrishnan *et al*., 2011). Herbs are been used in various countries to control shrimp and fish diseases, and successful results have been reported in Mexico, India, Thailand, and Japan (Auro de Ocampo and Jimenez, 1993; Dey and Chandra, 1995; Direkbusarakom *et al*., 1996a, 1996b; Logambal and Michael, 2000). For instance, aqueous extract of *Azadirachta indica* (neem) leaf has shown a positive result when used against *Aeromonas hydrophila* infection in common carp (Harikshnan *et. al.*, 2003).

1.4 Aim of the Study

To evaluate the antimicrobial effect of *Mangifera indica* (mango) kernel on *Aeromonas caviae* infection in *Clarias gariepinus* fingerlings.

1.5 Specific Objectives of the Study were:

1 To determine the antimicrobial effect of methanol and aqueous extracts of mango kernel on *Aeromonas caviae*.

2 To determine the serum antibacterial activity of *Mangifera indica* kernel against *Aeromonas caviae* infection in *Clarias gariepinus* fingerlings.

3 To determine the clinical manifestation of *A. caviae* infection in *Clarias gariepinus* fingerlings fed diet containing Mango Kernel.

4 To determine the effect of *Mangifera indica* kernel on some haematological parameters in *Clarias gariepinus*.

1.6 Research Hypotheses

1. *Mangifera indica* (mango) kernel does not have effects on *Aeromonas caviae* infection in *Clarias gariepinus* fingerlings.
2. *Mangifera indica* (mango) kernel does not have effect on some haematological parameters in *Clarias gariepinus* fingerlings.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fish Production

Global aquaculture production has continued to grow in the new millennium, albeit more slowly than in the 1980s and 1990s (FAO, 2012). In the course of half a century or so, aquaculture has expanded from being almost negligible to fully comparable with capture production in terms of feeding people in the world (FAO, 2012). Aquaculture has also evolved in terms of technological innovation and adaptation to meet changing requirements. World aquaculture production attained another all-time high in 2010, at 60 million tonnes (excluding aquatic plants and non-food products), with an estimated total value of US$119 billion (FAO, 2012). However, the stage of development and the distribution of aquaculture production remain imbalanced in all regions. A few developing countries in Asia and the Pacific, sub-Saharan Africa and South America have made considerable progress in aquaculture development in recent years and they are becoming significant or major producers in their respective regions. However, the disparity remains huge across the continents and geo-regions, as well as among countries of comparable natural conditions (FAO, 2003a). FAO estimates that fish provides 22% of the protein intake in sub-Saharan Africa. This share, however, can exceed 50% in the poorest countries especially where other sources of animal protein are scarce or expensive (FAO, 2003b).

In Nigeria, fish production is not only important as a source of rich protein, but it can also be use to enhance the welfare and income of the rural people and urban poor, that can directly promote their livelihood (CBN 2010b). Unfortunately, Nigeria is not producing enough fish for consumption; also, the fish industry is not providing the much needed

financial empowerment that the fish farmers need. According to the Food and Agriculture Organisation of the United Nations (FAO, 2009) there is a huge supply demand gap for fish and fishery products in Nigeria. According to the report, there is about 400,000 tons of supply in comparison to the 800,000 tonnes of demand. This makes Nigeria one of the largest importers of fish in the developing world, importing 600,000 metric tonnes annually (Moehl, 2003). It is therefore, necessary to ensure that improved fish production technologies that have been developed and disseminated are adopted, in order to increase fish production. The fishery industry is crucial to the world economy. The livelihood of millions of people worldwide are dependent on fish farming (FAO, 2009). Fish provides a rich source of protein for human consumption. The flesh of fish is also readily digestible and immediately utilizable by the human body, which makes it suitable and complementary for regions of the world with high carbohydrate diet, like Africa (FAO, 2005).

2.1.1 Biology and history of African catfish

The freshwater clariids are one of the 37 catfish families within the order Siluriformes (Teugels, 1986). Although some species are distributed in Syria, southern Turkey and throughout Southeast Asia, their diversity is greatest in Africa (Teugels, 1996; Teugels and Adriaens, 2003). Some of the generalized, fusiform species in Africa, such as *Clarias gariepinus are* broadly distributed, while the anguilliform species are restricted to swampy areas in the Nilo Sudan (Niger delta), the Lower Guinea and the Zaire (Congo River basin) ichthyological provinces (Poll, 1957; Roberts, 1975; Teugels, 1986; Teugels *et al*., 1990). Generalised clariids can be recognised by the heavy, dorso-ventrally flattened head, bearing four pairs of oral barbels. The eyes are generally very small. The upper and lower jaws bear patches of small, villiform teeth. The clariids have, as their commonly used names,

"airbreathing" or "walking catfish" suggest, several adaptations to improve their dispersal abilities. The posterior part of the branchial cavity houses the uniquely present suprabranchial respiratory organ, formed by arborescent structures (suprabranchial organ, other wise known as the arborescent or air–breathing organ) derived from the second and fourth gill arches (Greenwood 1961; Teugels and Adriaens, 2003). Their ability to perform aerial respiration enables them to migrate through deoxygenated swamps and pools, as well as migrate over land. Besides, the terrestrial migration is facilitated by the specialised locomotion pattern, in which pectoral spines and undulatory body movements are used. These adaptations lie at the base of the vast distribution of certain species, as for example the African species *Clarias gariepinus*, which has an almost Pan-African distribution and even occurs into the Middle East (Teugels, 1986).

2.1.2 Taxonomy

Although more than 100 different species of the Genus *Clarias* have been described in Africa, a systematic revision based on morphological, anatomical and biogeographical studies has been carried out by Teugels (1982a, 1982b, 1984), who recognized 32 valid species. The large African species which are of interest for aquaculture belong to the subgenus *Clarias*. In earlier systematic studies on the large African catfish species Boulenger (1911) as well as David (1935) recognized five species within this subgenus. Both authors used morphological criteria such as form of vomerine teeth, ratio of vomerine to premaxillary teeth band and the number of gill rakers. The five species were:

· *Clarias anguillarus*

· *Clarias senegalensis*

· *Clarias lazera*

· *Clarias mossambicus*

· *Clarias gariepinus*

In 1982 Teugels revised the subgenus *Clarias* and found only two species (*C. gariepinus* and *C. anguillaris*), if the number of gill rakers on the first branchial arch was considered; for *C. anguillaris* the number of gill rakers was rather low (14 to 40) while for *C. gariepinus* relatively high (20 to 100) (Teugels, 1986).

2.1.3 Nature and geographical distribution

The catfish genus can be defined as displaying an eel shape, having an elongated cylindrical body with dorsal and anal fins being extremely long (nearly reaching or reaching the caudal fin) with both fins containing only soft fin rays. The outer pectoral ray is in the form of a spine and the pelvic fin normally has six soft rays (FAO, 1996). The head is flattened, highly ossified, the skull bones (above and on the sides) forming a casque and the body is covered with a smooth, scaleless skin. The skin is generally darkly pigmented on the dorsal and lateral parts of the body. The colour is uniformly marbled and changes from greyish olive to blackish according to the substrate. On exposure to light the skin colour generally becomes lighter (FAO, 1996).

They have four pairs of unbranched barbels, one nasal, one maxillar (longest and most mobile) on the vomer and two mandibulars (inner and outer) on the jaw (FAO, 1996). Tooth plates are present on the jaws as well as on the vomer. The major function of the barbels is for prey detection (FAO, 1996).

A supra-branchial or accessory respiratory organ, composed of a paired pear-shaped air-chamber containing two arborescent structures is generally present. These arborescent or

cauliflower-like structures located on the fourth branchial arcs, are supported by cartilage and covered by highly vascularised tissue which can absorb oxygen directly from the atmosphere (Moussa, 1956). Since the air chamber communicates directly with the pharynx and the gill-chamber, this accessory air breathing organ allows the fish to survive out of water for many hours or for many weeks in muddy marshes (FAO, 1996).

2.1.4 Habitat

Clarias species inhabit calm (undisturbed) waters from lakes, streams, rivers, swamps to floodplains, many of which are subject to seasonal drying. The most common habitats frequented are floodplain swamps and pools in which the catfish can survive during the dry seasons due to the presence of their accessory air breathing organs (Bruton, 1979a; Clay, 1979).

2.1.5 Natural food and feeding

Although numerous studies on the food composition of *C. gariepinus* have been carried out, a consistent pattern has not emerged and they are generally classified as omnivores or predators. Micha (1973) examined catfishes from the River Ubangui (Central African Republic) and found that *C. lazera* (*C. gariepinus*) feed mainly on aquatic insects, fish and higher plant debris. They have been found to feed on terrestrial insects, molluscs and fruits (FAO, 1996).

Similarly, Bruton (1979b) found that catfish in Lake Sibaya (South Africa) fed mainly on fish or crustacea, and that terrestrial and aquatic insects were an important part of the diet of juvenile and adult fish which inhabit shallow areas. However, molluscs, diatoms, arachnids, and plant debris were the minor food items consumed in this lake. Munro (1967)

studied the feeding habits of *C. gariepinus* in Lake McIIwaine (Zimbabwe) and found that feed composition changed as fish became larger; Diptera, particularly chironomid pupae, predominating in the diet of the smaller fish and becoming progressively less important with increasing size. Zooplankton became more important with increasing fish size and predominated in the diet of the largest fish (FAO, 1996). Most of the minor food groups also showed a progressive increase or decrease in importance in relation to increasing size. The greater importance of zooplankton in the diet of large fish was believed to be due to the increased gape and number of gill rakers of the larger fish (Jubb, 1961; Groenewald, 1964); presumably resulting in more efficient filter feeding, Spataru *et al.* (1987) studied the feeding habits of *C. gariepinus* in Lake Kinneret (Israel) and found that preyed fish were the most abundant food component (81%) and constituted the highest biomass.

C. gariepinus as a slow-moving omnivorous predatory fish which feeds on a variety of food items from microscopic zooplankton to fish half its length or 10% of its own body weight (FAO, 1996). In order to feed on this wide variety of food organisms in different situations *C. gariepinus* is equipped with a wide array of anatomical adaptations for feeding under low visibility (Bruton, 1979b) including:

- A wide mouth capable of considerable vertical displacement for engulfing large prey or large volumes of water during filter feeding.

- A broad band of recurved teeth on the jaws and pharyngeal teeth preventing prey from escaping.

- An abundant network of sensory organs on the body, head, lips and circumoral barbels. These barbels are extensively used for prey detection and fixation. Hecht and Applebaum

(1988) found that *C. gariepinus* with barbels were 22.6% more efficient at catching prey than those without. This would indicate that tactile behaviour is important in the prey catching processes.

- A wide, rounded, caudal fin, typical of fish which ambush their prey.

- Long gill rakers on the five branchial arches.

- A short and dilatable oesophagus which opens into a distinct muscular stomach (mechanical digestion) and a simple thin walled intestine.

Slow, methodical searching is the normal predatory tactic of *C. gariepinus*, with catfish grasping their prey by suction; a negative pressure (suction) being created by a sudden increase of the bucco-pharyngeal chamber (FAO, 1996).

An important aspect of predation by *C. gariepinus* is their ability to switch feeding from one type of prey to another. In Lake Sibaya (South Africa), catfish ignore (or cannot catch) fish prey during daylight and feed mainly on invertebrates, which are abundant and relatively easy to catch. By contrast, at night, when fish prey become more vulnerable, they switch their feeding habits to fish prey (Bruton, 1979b). In general, fish prey provides far more energy per unit weight than other sources of feed (prey items). However, switching feeding habits relies on the existence of atleast two alternate abundant preys.

2.1.6 Natural reproduction

The male and females of *C. gariepinus* can be easily recognized as the male has a distinct sexual papilla, located just behind the anus which is absent in females. *C. gariepinus* shows

a seasonal gonadal maturation which is usually associated with the rainy season (De Graaf *et al*., 1995). The maturation processes of *C. gariepinus* are influenced by annual changes in water temperature and photoperiodicity and the final triggering of spawning is caused by a rise in water level due to rainfall (De Graaf *et al*., 1995).

An example of the maturation and spawning cycle of *C. gariepinus* in Lake Victoria (Kenya) indicates that reproduction starting in March just after the start of the first heavy rains (as indicated by the decrease in the Gonado Somatic Index, G.S.I.). Natural reproduction is generally completed in July with the G.S.I. remaining low until November, thereafter the oocytes gradually maturing and becoming ripe again in March (De Graaf *et al*., 1995). Spawning usually takes place at night in the shallow inundated areas of rivers, lakes and streams. Courtship is usually preceded by highly aggressive encounters between males. Courtship and mating takes place in shallow waters between isolated pairs of males and females (Owiti and Dadzie, 1989). The mating posture, a form of amplexus (the male lies in a U-shape curved around the head of the female) is held for several seconds. A batch of milt and eggs is released followed by a vigorous swish of the female's tail to distribute the eggs over a wide area. The pair usually rest after mating (from a few seconds to up to several minutes) and then usually resume mating (Bruton, 1979a). There is no parental care for ensuring the survival of the catfish offspring except by the careful choice of a suitable site. Development of eggs and larvae is rapid and the larvae are usually capable of swimming within 48-72 hours after fertilization at 23-28^{o}C .

2.2 *Mangifera indica* L. (Mango)

Mangifera indica L. (Mango) is a member of the Anarcadiaceae family which comprises more than 70 genera. Historical records suggest that its cultivation as a fruit tree originated

in India around 4000 years ago. In the early period of domestication, mango trees probably yielded small fruits, but folk selection of superior seedlings over many hundreds of years would have resulted in the production of larger fruits (Mukherjee, 1997). The genus *Mangifera* contains several species that bear edible fruit. Most of the fruit trees that are commonly known as mangos belong to the species *Mangifera indica*. The other edible *Mangifera* species generally have lower quality fruit and are commonly reffered to as wild mangos (Bally, 2006). *Mangifera indica* is a large evergreen tree, reaches 10 – 45 m high with a strong trunk and heavy crown. Native from tropical Asia, it has been introduced wherever the climate is sufficiently warm and damp and is now completely naturalized in many parts of tropics and subtropics (Ross, 1999). Although grown widely, mangos prefer a warm, frost-free climate with a well defined winter dry season. Rain and high humidity during flowering and fruit development reduces fruit yields. The tree generally flowers in mid- to late winter, with fruit maturing in the early to mid-summer months. The canopy is evergreen with a generally spreading habit. The heavy canopy of the mango is a source of shelter and shade for both animals and humans (Mukherjee, 1997).

2.2.1 Origin and distribution

Mango tree is a native of Southern Asia, especially Eastern India, Burma and the Andaman Islands, *M. indica* has been cultivated, praised and even revered in its homeland since ancient times. Buddhist monks are believed to have taken the plant on voyages to Malaya and Eastern Asia in the 4th and 5th Centuries BC (Morton, 1987). Persians are said to have taken mangoes to East Africa around the 10th Century AD (Morton, 1987). The fruit was grown in the East Indies before the earliest visits of the Portuguese who apparently introduced it to West Africa and Brazil in the early 16th Century. *M. indica* was then

carried to the West Indies, being first planted in Barbados about 1742 and later in the Dominican Republic; it reached Jamaica in about 1782 and, early in the 19th Century, reached Mexico from the Philippines and the West Indies (Morton, 1987). In this day and age, *M. indica* resides in most tropical biotopes in India, Southeast Asia, Malaysia, Himalayan regions, Sri Lanka, Africa, America and Australia (Calabrese, 1993; Kirtikar and Basu, 1993; Sahni, 1998).

2.2.2 Taxonomy and variety

The genus *Mangifera* belongs to the order Sapindales, Anacardiaceae family. Hundreds of *M. indica* cultivars are distributed throughout the world. The highest diversity occurs in Malaysia, particularly in peninsular Malaya, Borneo and Sumatra, representing the heart of the distribution range of the genus (Bompard and Schnell, 1997). Asia and India present over 500 classified varieties of which 69 are mostly restricted to tropical regions. *M. Indica* is one of the most widespread fruit trees in Western Africa, where 4 categories of mango varieties can be distinguished: (i) varieties of local or polyembryonic mangoes (mangots and Number One); (ii) first monoembryonic varieties propagated by grafting; (iii) the Floridian varieties, also monoembryonic and propagated by grafting, introduced later and used either for export, or for the regional markets (iv) Precocity (Rey *et al*., 2004).

Taxonomy of *Mangifera indica* Linn.

Division - Magnoliophyta

Class - Magnoliopsida

SubClass- Rosidae

Order - Sapindales

Family - Anacardiaceae

Genus - *Mangifera*

Species – *indica*

2.2.3 Size

Mangoes are long-lived evergreen trees that can reach heights of 15–30 m (50–100 ft). Most cultivated mango trees are between 3 and 10 m (10–33 ft) tall when fully mature, depending on the variety and the amount of pruning. Wild, non-cultivated seedling trees often reach 15 m (50 ft) when found in favorable climates, and they can reach 30 m (100 ft) in forest situations. The trees can live for over 100 years and develop trunk girths of over 4 m (13 ft) (Ross, 1999).

2.2.4 Canopy

Mango tree typically branch 0.6 – 2 m (2 – 6.5 ft) above the ground and develop an evergreen, dome-shaped canopy. Variability in canopy shape and openness occurs among varieties and with competition from other trees. Mangoes grown in heavily forested areas branch much higher than solitary trees and have an umbrella-like form (Rey *et al*., 2004).

2.2.5 Roots

Mango tree have a long taproot that often branches just below ground level, forming between two and four major anchoring taproots that can reach 6 m (20 ft) down to the water table. The more fibrous finer roots (feeder roots) are *Mangifera indica* (mango) found from the surface down to approximately 1 m (3.3 ft) and usually extend just beyond the canopy diameter. Distribution of the finer roots changes seasonally with the moisture distribution in the soil (Bally, 2006).

2.2.6 Flowers

Mango flowers are born on terminal inflorescences (panicles) that are broadly conical and can be up to 60 cm long on some varieties. Inflorescences usually have primary, secondary, and tertiary pubescent, cymose branches that are pale green to pink or red and bear hundreds of flowers (Bally, 2006). The mango has two flower forms, hermaphrodite and male, with both forms occurring on the same inflorescence. The ratio of hermaphrodite to male flowers on an inflorescence varies with variety and season and is influenced by the temperature during inflorescence development. Hermaphrodite flowers are small (5–10 mm) with four to five ovate, pubescent sepals and four to five oblong, lanceolate, thinly pubescent petals. Only one or two of the four to five stamens that arise from the inner margin of the disc are fertile. The single ovary is born centrally on the disc with the style arising from one side. The disc is divided into a receptacle of four or five fleshy lobes that forms the nectaries. The male flowers are similar to the hermaphrodite flowers but are without the pistil, which has been aborted (Calabrese, 1993; Kirtikar and Basu, 1993; Sahni, 1998).

2.2.7 Leaves

The leaves are simple, without stipules, and alternate, with petioles 1–12 cm long. The leaves are variable in shape and size but usually are oblong with tips varying from rounded to acuminate. Leaf form differs among varieties but is more consistent within a variety. However, a range of leaf sizes can be seen on a single tree (Bally, 2006). Mature leaves are dark green with a shiny upper surface and glabrous lighter green lower surface. New leaves emerge in flushes (episodic growth spurts) of 10–20 leaves. Leaves emerge green, turning tan-brown to purple during leaf expansion and then gradually changing to dark green as the

leaves mature. The color of the young, expanding leaf varies with variety and can be from light tan to deep purple; this can be used as a distinguishing character among varieties (Calabrese, 1993; Kirtika, and Basu, 1993; Sahni, 1998).

2.2.8 Fruit

Mango fruit is classed as a drupe (fleshy with a single seed enclosed in a leathery endocarp). Fruits from different varieties can be highly variable in shape, color, taste, and flesh texture. Fruit shapes vary from round to ovate to oblong and long with variable lateral compression. Fruits can weigh from less than 50 g (0.35 lb) to over 2 kg (4.4 lb) (Bompard and Schnell, 1997). The fruit has a dark green background color when developing on the tree that turns lighter green to yellow as it ripens. Some varieties develop a red background color at fruit set that remains until the fruits ripen. In addition to the background color, many varieties also have an orange, red, or burgundy blush that develops later in the fruit development, when the rind is exposed to direct sunlight. The mesocarp is the fleshy, edible part of the fruit that usually has a sweet and slightly turpentine flavor. When ripe, its color varies from yellow to orange and its texture from smooth to fibrous (Bompard and Schnell, 1997).

2.2.9 Seed

Mango varieties can be classified as having either monoembryonic or polyembryonic seed embryos. In monoembryonic varieties, the seed contains only one embryo that is a true sexual (zygotic) embryo (Bompard and Schnell, 1997). Monoembryonic seeds are a cross between the maternal and paternal (pollen) parents. Fruit from monoembryonic seedlings will often vary from the parent trees, so propagation by grafting is used to produce true-to-type monoembryonic trees. Polyembryonic seeds contain many embryos, most of which are

asexual (nucellar) in origin and genetically identical to the maternal parent. Polyembryonic seeds also contain a zygotic embryo that is the result of cross-pollination (Bompard and Schnell, 1997). The monoembryonic seedling usually has less vigor than a nucellar seedling for use as a rootstock. In some varieties this is reversed and the zygotic seedling is the most vigorous. The occurrence of off-types in orchards is often attributed to use of zygotic seedlings (Ross, 1999).

2.2.10 Pharmacology of *M. indica* seed (kernel)

Mango (*Mangifera indica*) is one of the most important tropical fruits with a global production exceeding 35 million tons and in Egypt, 450,000 tons of mango fruits were produced in 2009 (FAO, 2009). A preliminary study showed that the mango seed represents from 20% to 60% of the whole fruit weight, depending on the mango variety, and the kernel inside the seed case represents from 45% to 75% of the whole seed (Maisuthisakul and Gordon, 2009; Kim *et al.*, 2010). Despite the fact that mango seed kernel contains 6% protein, 11% fat, 77% carbohydrate, 2% crude fiber and 2% ash, the seeds are considered to be a waste (Abdalla *et al.*, 2006). Several studies have shown that mango seed kernels contain various phenolic compounds and can be a good source of natural antioxidants (Abdalla and Hammam, 2007; Puravankara *et al.*, 2000). Mango seed kernels have previously been shown to possess potent antioxidant activities which could be ascribed to their phenolic contents (Soong & Barlow, 2004). The antimicrobial activity of mango kernel extract is certainly related to seven major phenolic compounds assumed to be mainly ellagic acid, gallic acid, opigenin-oglucoside, mangiferin, methylgallate and pentagalloyl glucose (Hammam, 2007). Mango kernel extract reduced total bacterial count, inhibited coliforms growth, showed remarkable antimicrobial activity against *E. coli* strain and

extends shelf life of pasteurized cow milk (Abdallah and El-Hamahmy, 2007). The crude methanolic extract of mango seed kernel at a concentration of 100 mg/ml was found to have potential antimicrobial activity against *MRSA* and *E. coli* compared to *V. vulnificus* (Kim *et al.,* 2010). Extracts from mango kernel showed superoxide anion scavenging activity in a cell-free system (Saito *et al*., 2008), suggesting one possible bioactivity by antioxidant mechanism. Mangiferin, a xanthone present in mango, when administered at a dose of 100 mg/kg to rats subjected to experimental periodontitis, demonstrated an anti-inflammatory property, accelerating the processes of repairing and healing injured tissues (Carvalho *et al*., 2009). Among the rural folks the mango seed kernels are used traditionally as antimicrobials against gastric pathogens especially for the infants (Kim *et al.*, 2010). The mango seed extract showed interesting antibacterial activity against both gram positive and gram negative bacteria as determine by disc diffusion method. The most sensitive pathogenic strain inhibited by all extracts was *Pseudomonas aeruginosa* (Khammuang and Santhima, 2011). A wide range of biological activities, including antibacterial, antithrombotic, vasodilatory, anti-inflammatory, and anticarcinogenic effects mediated by different mechanisms, are associated with flavonoids (Middleton *et al*., 2000). Inverse relations were found between the dietary intake of some flavonoids and incidence of several chronic diseases (Knekt *et al*., 2002; Knouhouser, 2004). GA, EA and gallates have been determined in mango kernel by paper chromatography (Puravankara *et al*., 2000). Gallotannins and condensed tannin-related polyphenols were also shown to be present in mango kernel using thin-layer chromatography (Arogba, 2000).

2.2.11 Phytochemistry of *M. indica* kernel

Gallotannins and condensed tannin-related polyphenols were reported to be present in mango kernels (Arogba, 1997). In addition, polyphenols from dry mango kernel meal were found to contain tannic acid, gallic acid, and epicatechin in the ratio 17:10:1, respectively (Arogba, 2000). Abdalla and Hammam (2007) have characterized the phenolic compounds in Egyptian mango seed kernels. The components included tannins, gallic acid, coumarin, ellagic acid, vanillin, mangiferin, ferulic acid, cinammic acid and unknown compounds. The presence of the phenolic compounds glucogallin and gallotanin in mango flesh and kernels, and mangiferin, isomangiferin, homomangiferin, fisetin, quercetin, isoquercitin, astragalin, gallic acid, methyl gallate, digallic acid, β-glucogallin, and gallotanin in leaves, twigs, seeds, and fruits of 20 local varieties was described already in 1971 (El Ansari *et al.*, 1971). Peels and kernels contain large amounts of extractable phenolics, and there are varietal differences in their contents (Ribeiro, 2006). A wide pattern of phenolic compounds has been described in the flesh, peels, and kernels of mangoes (Schieber *et al*., 2001). The flavonols (quercetin, kaempferol, and rhamnetin) are present mostly as O-glycosides, whereas mangiferin is a C-glycoside and occurs both in its non-esterified form and conjugated with gallic acid. Quercetin and kaempferol belong to the flavonoid class and have been receiving great attention as bioactive compounds for many years.

2.3 Aeromonads

2.3.1 Taxonomy

Taxa in the genus *Aeromonas* have undergone a number of significant nomenclature changes in the phylogenetic evolution of this genus (Carnahan and Altwegg, 1996). In the mid to late 1970s, most aeromonads were viewed as belonging to one of two major groups.

Strains that grow at 35˚C to 37˚C (mesophiles) and were responsible for a variety of human infections were commonly referred to as *Aeromonas hydrophila.* Psychrophilic strains that grew better at lower temperatures (22˚C to 28˚C) and primarily caused infections in fish, such as salmonids, were designated *Aeromonas salmonicida.* These two groups were phenotypically quite distinct and could easily be distinguished from one another on the basis of a number of properties, including optimal growth temperatures, motility, production of indole, and elaboration of a melanin like pigment on tyrosine agar (Popoff *et al.,* 1981). While the taxonomy of the psychrophilic strains has remained fairly stable with minor exceptions, the number of mesophilic species has increased over the past years. These changes have been pioneered by three groups of researchers at the Institute of Pasteur (Popoff and Veron, 1976; Popoff *et al.,* 1981), the Centers for Disease Control and Prevention (Hickman-Brenner *et al.*, 1987), and the University of Maryland (Allen *et al.*, 1983) all in the USA. In the mid-1970s, with the study of Popoff and Ve´ron (1976), followed by a series of articles employing DNA-DNA reassociation techniques, 12 initial DNA or hybridization groups (HGs) were established. Certain HGs, such as HG 1and HG 3, were found to respectively contain the type strains of *A. hydrophila* and *A. salmonicida* and were therefore, immediately defined. Other groups, established prior to 1987, included *Aeromonas caviae* (HG 4), *Aeromonas media* (HG 5), and *Aeromonas sobria* (HG 7). The remaining HGs, or genomospecies or genomic species (Brenner *et al*., 1993), were unnamed at the time or their taxonomic status was uncertain. Since 1987, a number of new *Aeromonas* species have been proposed and include *A. veronii* (Hickman-Brenner *et al.*, 1987), *A. schubertii* (Hickman-Brenner *et al.*, 1988), *A. jandaei* (Carnahan *et al*., 1991), *A. trota* (Carnahan *et al*., 1991), *A. encheleia, A. bestiarum* (Ali *et al.,* 1996), and *A. popoffii* (Huys *et al.,* 1997). Two others (*Aeromonas ichthiosmia* and *A. enteropelogenes*) appear to

be subjective synonyms of previous published species (Schubert *et al.*, 1990; Collins *et al.*, 1993). Recently proposed taxa for inclusion in genus *Aeromonas* are *A. tecta* (Demarta *et al* ., 2008), *A. aquariorum* (Martinez-Murcia *et al.*, 2008), *A. bivalvium*(Minana-Galbis *et al.*, 2007), *A. sharmana* (Saha and Chakrabarti, 2006), *A. molluscorum* (Minana-Galbis *et al.*, 2007), *A. simiae* (Harf-Monteil *et al*., 2004) and *A. culicola* (Pinna *et al*., 2004)

2.3.2 Historical aspects of aeromonads

The history of the genus *Aeromonas* mirrors the chronicles of modern-day medical bacteriology, which spans over 100 years, from its birth as a recognized laboratory science in the late 19th and early 20th centuries through its evolution into the molecular postgenomic era (Janda and Abbott, 2010). The perception of the genus *Aeromonas* by the scientific community has likewise evolved over the same interval. Initially, aeromonads were recognized only as causing systemic illnesses in poikilothermic animals. Today, the genus *Aeromonas* is regarded not only as an important disease-causing pathogen of fish and other coldblooded species but also as the etiologic agent responsible for a variety of infectious complications in both immunocompetent and immunocompromised persons (Janda and Abbott, 2010). From the creation of the genus *Aeromonas* in 1943 through the mid-1970s, aeromonads could be broken down roughly into two major groupings, based upon growth characteristics and other biochemical features (Janda and Duffey, 1988). The mesophilic group, typified by *A. hydrophila*, consisted of motile isolates that grew well at 35 to 37°C and were associated with a variety of human infections. The second group, referred to as psychrophilic strains, caused diseases in fish, were nonmotile, and had optimal growth temperatures of 22 to 25°C. This group contained isolates that currently reside within the species *A. salmonicida*. Beginning in the mid-1970s and continuing for

almost 10 years thereafter, several groups, spearheaded an effort to redefine the mesophilic group based upon DNA relatedness studies. Over that span of time, DNA hybridization investigations revealed that multiple hybridization groups (HGs) existed within each of the recognized mesophilic species (*A. hydrophila*, *A. sobria*, and *A. caviae*) (Popoff *et al*., 1981; Fanning *et al.*, 1985). Unnamed HGs were represented by reference strains, since in each case they could not be separated unambiguously from each other by simple biochemical means. In general, there was consensus agreement on the first 12 HGs between the Institute of Pasteur and CDC. At a later time, when phenotypic markers were recognized that clearly separated these groups from one another, new species were proposed,such as *A. trota* (Voges-Proskauer [VP] negative, ampicillin susceptible), *A. schubertii* (D-mannitol negative), and *A. jandaei* (sucrose negative) (Carnahan, 1993).

The genus *Aeromonas* has taxonomically resided in the family Vibrionaceae since 1965 with two other genera that are pathogenic for human (*Vibrio* and *Plesiomonas*). This systematic classification scheme was convenient, if not genetically sound, as all three genera shared similar phenotypic features, ecosystems (water, fish, reptiles, and amphibia), and disease spectrums (gastroenteritis and septicemia) (Janda and Abbott, 1998). Although, sophisticated molecular techniques, including 16S rRNA sequencing, have indicated that these three genera are not closely related to each other on an evolutionary basis; plesiomonads are more closely related to the Enterobacteriaceae, and aeromonads are represented by a family of their own (Carnahan, 1993; Ruimy *et al.*, 1994).

2.3.3 Isolation of aeromonas

Transportation of *Aeromonas* at room temperature yields the greatest recovery. When specimens are transported at 4°C for 24 hours, colony counts decline and may only return

to its normal condition, if at all, after being held for several days at that temperature (Janda and Abbott, 2010). Aeromonads of clinical significance grow well on noninhibitory laboratory media used for culture of bacteria from sterile sites as well as on most enteric isolation media, with the exception of thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Although growth is not a problem on routine enteric isolation media (MacConkey, Xylose lysine deoxycholate sugar, Hekteon agar, Salmonella Shigella, and Deoxycholate citrate agar media), lactose-negative isolates must be differentiated from commonly isolated pathogens such as *Salmonella* and *Shigella*, or if the organism ferments lactose or sucrose, it may be assumed to be normal flora and be overlooked. However, cefsulodin-irgasan-novobiocin (CIN) agar, used for the isolation of *Yersinia*, has been found to support the growth of *Aeromonas* as well as *Plesiomonas shigelloides*, making this agar multifunctional and hence increasing its cost-effectiveness (Janda and Abbott, 2010). Like *Yersinia*, *Aeromonas* forms a bull's eye-like colony due to fermentation of D-mannitol, while *P. shigelloides*, which does not ferment D-mannitol, produces colourless colonies. Usually, *Citrobacter* specie are the only normal faecal flora that grow on CIN with any frequency, but their colony morphology is similar to that of *Yersinia* and *Aeromonas* (Janda and Abbott, 2010). Because of false-negative reactions due to acid produced by fermentation of D-mannitol, an oxidase test which readily separates *Aeromonas* from *Yersinia* and citrobacters cannot be performed directly from CIN agar (Janda and Abbott, 2010).

2.3.4 Biochemical appearance and identification

Aeromonas specie are oxidase-positive, facultatively anaerobic, gram-negative rods that grow readily on basic laboratory media such as Heart Infusion Agar (Janda and Abbott,

2010). Among species isolated from humans, >90% of strains produce β-hemolysis on sheep blood agar, with the exception of *A. popoffii* and *A. trota* (0% and 50%, respectively). Species of this genus (with the exception of *A. schubertii*) are considered to be indole positive, but there are some strains, particularly *A. caviae* strains, that remain negative after 7 days even when extracted with xylene (Janda and Abbott, 2010). Likewise, rare strains of *A. caviae* hydrolyze urea, a characteristic presumed to be negative in aeromonads. Identification of *Aeromonas* to the species level can be very challenging, and identification of strains from nonsterile sites may not be practical (Janda and Abbott, 2010). Very few clinical laboratories will be able to identify the clinically significant species of this genus beyond complexes or groups (i.e., *A. hydrophila* complex or *A. caviae* complex). Likewise, it can be difficult to separate *A. veronii* biovar sobria from *A. hydrophila* by using conventional biochemical tests. For the most part, the only other genera that they may be confused with are *Vibrio* and *Plesiomonas* (Janda and Abbott, 2010).

Aeromonas specie can be separated from vibrios by their ability to grow in nutrient broth (Difco) without NaCl supplementation, their inability to grow on TCBS agar, and their resistance to the vibriostatic agent 2,4-diamino-6,7-diisopropyl-pteridine (O129). Some strains of *V. cholerae* O1 and all strains of *V. cholerae* O139 are now resistant to O129, but their decarboxylase pattern is very different from that of aeromonads, except for *A. veronii* biovar veronii. A positive reaction in esculin or salicin and production of gas from glucose will identify the strain as *A. veronii* biovar veronii. The ability of aeromonads to grow on TCBS agar can vary depending on the manufacturer, but if growth is present, *Aeromonas* colonies usually range in size from very small (~1 mm) to pinpoint colonies (Janda and Abbott, 2010). Since the fermentable substrate in TCBS is sucrose

and *Aeromonas* spp. are variable sucrose fermenters, colonies can appear either green or yellow, depending on the species. It also can be very difficult to differentiate *A. caviae* from some strains of *Vibrio fluvialis*. The latter agent, although usually requiring salt, can on occasion grow in a variety of media without NaCl supplementation, and the zone around the O129 disk can be very small, approaching 6 mm. Fermentation of cellobiose but not D-arabinose will set *A. caviae* strains apart. *P. shigelloides* is positive for lysine and ornithine decarboxylases and for arginine dihydrolase and ferments *myo*-inositol, characteristics that are not found in any aeromonads (Janda and Abbott, 2010).

2.3.5 Aeromonas and ecosystem

Aeromonads are essentially ubiquitous in the microbial biosphere. They can be isolated from virtually every environmental niche where bacterial ecosystems exist. These include aquatic habitats, fish, foods, domesticated pets, invertebrate species, birds, ticks and insects, and natural soils, although extensive investigations on the latter subject are lacking (Janda and Abbott, 2010). The vast panorama of environmental sources from which aeromonads can be encountered lends itself readily to constant exposure and interactions between the genus *Aeromonas* and humans. The relative environmental distributions of *Aeromonas* species in selected settings with their distribution pattern, as currently known, are presented in Table 2.1 (Janda and Abbott, 2010). Earlier studies have indicated that three *Aeromonas* genomospecies (*A. hydrophila*, *A. caviae*, and *A. veronii* bv. sobria) are responsible for the vast majority (≥85%) of human infections and clinical isolations attributed to this genus (Janda and Abbott, 1998). The same pattern observed clinically appears to repeat itself in most environmental samples, with *A. salmonicida* included as a predominant species in fish and water samples. In some studies, less frequently

encountered species have been found to predominate in environmental samples, such as *A. schubertii* in organic vegetables (MacMahon and Wilson, 2001). For newly described species such as *A. aquariorum* and *A. tecta*, no data exist on their relative distributions in the environment outside their initial taxonomic description, and extremely limited data are available on many other taxa described since 2004. Finally, the techniques and methods used to identify *Aeromonas* isolates to the species level vary considerably from one study to another (Janda and Abbott, 2010).

Table 2.1: Relative distributions of *Aeromonas* species in animals and the environment.

	Vertebrates		Invertebrates			Water		
Species	Primates	Others	Molluscs	Arthropods	Others	Fresh	Saline	Foods
A. allosaccharophila	±	±	0	0	0	0	0	0
A. aquariorum	0	±	0	0	0	±	0	0
A. bestiarum	+	±	±	0	0	++	0	0
A. bivalvium	0	0	±	0	0	0	0	0
A. caviae	+++	+++	++	++	0	++	+++	+++
A. encheleia	0	++	±	0	0	+	0	0
A. eucrenophila	±	+	0	0	0	+	0	0
A. hydrophila	+++	+++	±	±	0	+++	++	++
A. jandaei	+	++	±	0	+	±	0	0
A. media	+	0	0	0	0	+	0	0
A. molluscorum	0	0	±	0	0	0	0	0
A. popoffii	±	0	0	0	0	+	0	0
A. salmonicida	+	+++	0	0	0	++	0	0
A. schubertii	+	±	0	0	0	0	0	±
A. simiae	±	0	0	0	0	0	0	0
A. sobria	0	++	0	0	0	±	0	0
A. tecta	±	±	0	0	0	0	0	0
A. trota	+	0	0	0	0	0	0	±
A. veronii	+++	++	0	±	++	±	++	++

0, not reported to date; ±, rare reports; +, uncommon; ++, common; +++, predominant species.
(Allen *et al.*, 1983; Borrell *et al*, 1998; Demarta *et al*, 2008; Huys *et al.*, 1997; Laufer *et al*., 2008; Martinez-Murcia *et al.*, 2008; MacMahon and Wilson, 2001).

2.3.6 Diseases caused by aeromonas in fish

The role of aeromonads as causative agents of fish diseases has been known for decades, longer than their comparable role in causing systemic illnesses in humans. Two major groups of fish diseases are recognized. *A. salmonicida sensu stricto* causes fish furunculosis, particularly in salmonids (Janda and Abbott, 2010). The disease has several presentations, ranging from an acute form characterized by septicemia with accompanying hemorrhages at the bases of fins, inappetence, and melanosis to a subacute to chronic variety in older fish, consisting of lethargy, slight exophthalmia, and haemorrhaging in muscle and internal organs (Austin and Adams, 1996). Mesophilic species (*A. hydrophila* and *A. veronii*) cause a similar assortment of diseases in fish, including motile *Aeromonas* septicemia (haemorrhagic septicemia) in carp, tilapia, perch, catfish, and salmon, red sore disease in bass and carp, and ulcerative infections in catfish, cod, carp, and goby (Joseph and Carnahan, 1994). Mesophilic *Aeromonas* species, most notably *A. hydrophila*, have been linked to major die-offs and fish kills around the globe over the past decade, resulting in enormous economic losses. These die-offs included over 25,000 common carp in the St. Lawrence River in 2001, 820 tons of goldfish in Indonesia in 2002, resulting in a $37.5 million loss (Monette *et al.*, 2006).

2.3.7 Pathology

Motile aeromonads cause diverse pathologic conditions that include acute, chronic, and covert infections. Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiologic condition of the host, and the degree of genetic resistance inherent within specific populations of fishes (Joseph and Carnahan, 1994). Motile aeromonads differ

interspecifically and intraspecifically in their relative pathogenicity or their ability to cause disease. Under controlled laboratory conditions, De Figueredo and Plumb (1977) found that strains of motile aeromonads isolated from diseased fish were more virulent to channel catfish than were those isolated from pond water. Lallier *et al.* (1981) performed studies on rainbow trout *(Oncorhyncus mykiss*, formerly *Salmo gairdneri)* to compare the relative virulence of *A. hydrophila* and *A. sobria*, as taxonomically described by Popoff and Vernon (1976). The results indicated that strains of *A. hydrophila* isolated from either healthy or diseased fish were more virulent than strains of *A. sobria.* (Boulanger *et al*., 1977). Paniagua *et al.* (1990), collected aeromonad isolates along the River Porma, Leon Province (Spain) and found that the isolates belongs to three species; *A. hydrophila* (n=74 strains), *A. sobria* (n =11 strains), and *A. caviae* (n = 12 strains). The workers also observed that 72.02% of *A. hydrophila* isolates and 63% of *A. sobria* isolates were virulent in fish when challenged through intramuscular route, but all of the strains of *A. caviae* were avirulent. Pathologic conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, haemorrhagic septicemia, red sore disease, red rot disease, and scale protrusion disease (Paniagua *et al.*,1990). In the acute form of disease, a fatal septicemia may occur so rapidly that fish die before they have time to develop major signs but a few gross lesions of the disease. When clinical signs of infection are present, affected fish may show exophthalmia, reddening of the skin, and an accumulation of fluid in the scale pockets (Faktorovich, 1969). The abdomen may become distended as a result of an oedema and the scales may bristle out from the skin to give a “washboard” appearance. The gills may be haemorrhagic and ulcers may develop on the dermis. Ogara *et al*. (1998) noted severe eye pathology and heavy mortality among yearling and older rainbow trout accompanying a severe outbreak of

motile aeromonads septicemia. The condition at first affected one eye, progressed into the other eye, after which the orbits ruptured causing blindness and death. Similarly, Yambot and Inglis (1994) described an acute mortality among Nile tilapia in which the most apparent clinical signs included an opacity in one or both eyes, accompanied by exophthalmia and eventual bursting of the orbit. Motile aeromonads were isolated from the eyes, liver and kidneys of affected fish. Histopathologically, fish may exhibit epithelial hyperplasia in the foregut; leptomeningeal congestion in the brain, as well as a thrombosis and inflammation in the perisclerotic region and corneal epithelium of the eye (Fuentes and Perez, 1998). There may also be a severe bronchitis, as indicated by leukocytic infiltration and dilation of the central venous sinus (Grizzle and Kiryu, 1993). It was also noted that catfish with septicemic or latent infections had enlarged nuclei in the bronchial epithelium and that there was a significant correlation between the presences of these gill lesions and the severity of hepatic and pancreatic lesions. Rodriguez *et al*. (1993) further noted that there was an increase in bacterial acetylcholinesterase activity in the brain tissue of moribund fish. Systemic infections were characterized by diffuse necrosis in several internal organs and the presence of melanin-containing macrophages in the blood (Ventura and Grizzle 1988). Internally, the liver and kidneys are target organs of an acute septicemia. The liver may become pale or have a greenish colouration while the kidney may become swollen and friable. These organs are apparently attacked by bacterial toxins and lose their structural integrity (Huizinga *et al*. 1979). Even when tissue damage in the liver and kidneys is extensive, the heart and spleen are not necessarily damaged. However, splenic ellipsoids are often centers of intense phagocytic activity by macrophages. Bach *et al.* (1978) observed pathological changes in the spleens of fish injected with virulent *A. hydrophila*, whereas fish infected orally showed little or no splenic involvement. Bacteria

were present within the reticular sheaths of the ellipsoids, where intense phagocytic activity by macrophages occurred. Phagocytized bacteria divided intracellularly and extracellularly and destroyed the endothelial and reticular cells of the ellipsoids. The lower intestine and vent, which sometimes protrude from the body, are often swollen, inflamed, and haemorrhagic. Additionally, the intestine is devoid of food and may be filled with a yellow mucus-like material. Chronic motile aeromonads infections manifest themselves primarily as ulcerous forms of disease, in which dermal lesions with focal haemorrhage and inflammation are apparent. Both the dermis and epidermis are eroded and the underlying musculature becomes severely necrotic (Huizinga *et al.*, 1979). Inflammatory cells are usually lacking in the necrotic musculature, whereas the adjacent epidermis undergoes hyperplasia that results in a raised margin. At this stage, the infection has usually become systemic and pinpoint hemorrhages (petechiae) may occur throughout the peritoneum and musculature. Fish with only cutaneous infections may have several types of concealed lesions including increased amounts of lipofuscin and haemosiderin in the liver and spleen; however, most visceral organs are not necrotic (Ventura and Grizzle 1988). Recently Hazen *et al.* (1978b) re-examined the etiology of red sore disease and found that *A. hydrophila* was present in 96% of the initial lesions on fish, whereas *Epistylis* was present in only 35% of such lesions. Furthermore, electron microscopy showed that *Epistylis* lacked structures that produced lytic enzymes and, therefore, could not initiate the development of lesions. This study strongly suggested that *A. hydrophila* is indeed the primary aetiological agent of red sore disease and that *Epistylis* is a secondary pathogen that rapidly colonizes the dermal lesions initiated by bacterial proteolytic enzymes. In frogs and other amphibians, *A. hydrophila* infections cause distension of capillaries on the ventral surface of the legs and abdomen, giving them the red colouration that is the source of the name of the disease; red

leg. Outbreaks of aeromonads septicemia in frogs and warm water fishes usually occur in the spring and coincide with an increase in water temperature. Resistance to disease is lowered at this time because aquatic organisms are often anaemic and have a substantial decrease in serum proteins resulting from periods of dormancy and starvation that occurred during the winter. Huizinga *et al.* (1979) also indicated that rising water temperatures increased metabolism, decreased overall condition, and stressed the fish. Stressed fish increased production of corticosteroids, which in turn increased their susceptibility to infection.

2.3.8 Virulence factors

The ability of a pathogen to locate, attach to, and subsequently infect a susceptible host is a primary step in the development of disease. Consequently, factors produced by motile aeromonads, which can facilitate contagion, are important elements of bacterial virulence. Infact, motile aeromonads that have been taken from lesions on diseased fish have been shown to have a greater chemotactic response to skin mucus than isolates that were obtained as free-living organisms from pond water (Hazen *et al.* 1982). Ascencio *et al.* (1998) have shown that *A. hydrophila*, *A. caviae*, and *A. sobria* can actually adhere to animal cell lines that have mucous receptors. These workers also determined that the proportion of *A. hydrophila* strains which bound various mucins was significantly greater than the proportions of *A. caviae* and *A. sobria* that acted similarly. Trust *et al.* (1980) also indicated that *A. hydrophila* had adhesive agglutination characteristics which facilitated attachment to eukaryotic cells. Electron microscopy further demonstrated that motile aeromonads produce fimbriae (pili) that facilitate adhesion but these structures were common to cells regardless of their virulence (Del Corral *et al.,* 1990). In addition to

adhesins, Dooley and Trust (1988) characterized a tetragonal surface protein array consisting of a 52 kD protein from virulent isolates of *A. hydrophila* . This S-layer was also reported by Ford and Thune (1991) among motile aeromonads that they isolated from clinically diseased catfish. The existence of such layers structurally permeate bacterial cell membranes which generally increases cellular hydrophobicity. The increased surface tension enhances resistance of the bacterium to complement-mediated serum lysis and phagocytosis by leukocytes. Byers *et al*. (1986) have also shown that *A. hydrophila* can produce siderophores that confer resistance against the ability of serum transferrin to inhibit bacterial growth. Many studies have attempted to further delineate the virulence mechanisms of motile aeromonads. Kou (1973) found that many of the virulent, avirulent, and attenuated aeromonads that he studied possessed haemorrhagic factors and lethal toxins. The virulent bacteria had quantitatively more toxic potential than did their avirulent or attenuated counterparts. Olivier *et al*. (1981) indicated that both *A. hydrophila* and *A. sobria* produced enterotoxins, dermonecrotic factors, and hemolysins. Although both species produced haemolysis on blood agar plates at 30°C, only *A. hydrophila* did so at 10°C. Since these workers, worked with salmonid fish, they suggested that the haemolysis of red blood cells by *A. hydrophila* at temperatures comparable to those of the water in which fish live may at least partially account for the difference in virulence between A. *hydrophila* and *A. sobria*. Enterotoxins, haemolysins, proteases, haemagglutinins, and endotoxins produced by this complex of bacterial organisms have been the subject of much research (Cahill, 1990).

However, the composite nature of virulence observed both inter- and intra-specifically among the motile aeromonads may be best defined by looking at synergistic relationships between virulence factors. For example, Rigney *et al*. (1978) found that individual

injections of either endotoxin or haemolysin alone did not produce clinical pathology in frogs. However, when both the endotoxin and haemolysin were injected in the same inoculum, frogs exhibited pathology that mimicked clinical signs of red leg disease. Thune *et al.* (1982a) also found that channel catfish were tolerant to injections of endotoxin alone at concentrations up to 400 μg endotoxin per 7.2 g of fish. However, an extracellular cell-free extract of a spent culture medium had an LD_{50} value of 15.7 μg protein within 48 h after its injection into 7.2 g fish. Thune *et al.* (1982b) later showed that this extract was proteolytic but not haemolytic. Two proteases were further refined from this extract; one was heat labile and had a median lethal concentration LC_{50} concentration of 18 μg of protein per gram of fish, and the other was heat stable and had an LC_{50} concentration of 3 μg of protein per gram of fish. Allan and Stevenson (1981) also found hemolytic and proteolytic activities in crude extracellular preparations from *A. hydrophila.* These workers indicated that aeration increased growth rates, cell yield, and the amount of proteolytic activity. Proteolytic activity was reduced, however, when cultures were incubated at 37°C. Furthermore, extracts from a protease deficient mutant were more toxic to fish than were similar extracts from isogenic wild strains. Because the extract from the protease deficient mutant had a fivefold increase in haemolytic activity, Allan and Stevenson (1981) concluded that haemolysin, not protease, was the principal virulence factor of *A. hydrphila.* The results of other studies, however, might caution against such conclusions. Although Rogulska *et al.* (1994) found that haemolytic activity was high in 93% of very pathogenic strains of *A. hydrophila* and the activity was also high in 87.5% of pathogenic *A. sobria.* Measurement of haemolytic activity alone was not the decisive characterstic in terms of isolate virulence because even some (15%) avirulent *A. hydrophila* isolates were haemolytic. Additional measurements showed that a combination of high haemolytic and

high proteolytic activity, which was detected in 90% of virulent *A. hydrophila* strains and 87.5% of the *A. sobria* strains, may be a better measure of total virulence. Only one non-pathogenic strain of *A. hydrophila* had high levels of both activities. Rodriguez *et al.* (1992) have purified a metalloprotease, a serine protease and a haemolysin from culture supernatants of *A. hydrophila*. Each of these factors have lethal activity for rainbow trout. The metalloprotease had a molecular weight of 38 kd, was stable at 56°C for 10 min, had no cytotoxic activity but produced LC_{50} value of 150 ng/g of trout. The serine protease had a molecular weight of 22 kD, was also stable at 56°C for 10 min, possessed cytotoxic activity and also had an LC_{50} value of 150 ng/g of trout. The haemolysin was alpha -hemolytic and had a molecular weight of 68 kD and produced an LC_{50} value of 2 μg/g of trout. The haemolysin was stable at 56°C for 20 min and at 60°C for 10 min. Because the haemolysin possessed esterase activity on beta -naphthyl acetate, Rodriguez *et al.* (1993) suggested that the compound may be distinct from either alpha or beta - haemolysin. Casc'on *et al.* (2000) have cloned a gene encoding elastolytic activity, *ahyB*, from *Aeromonas hydrophila*. *AhyB* is synthesized as a pre-proprotein which is further processed into a mature protease and a C-terminal propeptide. The protease hydrolyzed casein and elastin and showed a high sequence similarity to other metalloproteases. Results of a molecular analysis, which was conducted by Zhang *et al.* (2000) to underscore genetic differences between virulent and avirulent isolates, confirms that virulence among the motile aeromonads depends upon a multiplicity of factors. Their results showed that 22 DNA fragments were present in most of the virulent strains and that these genes encoded for five known virulence factors of *A. hydrophila* including haemolysin (hlyA), protease (oligopeptidase A), outer-membrane protein (Omp), multidrug-resistance protein and histone-like protein (HU-2). These same fragments were mostly absent in the avirulent isolates that were examined.

2.3.9 Serology and vaccination

Motile aeromonads are one of the most taxonomically and antigenically diverse groups of bacteria pathogenic to fish. The amount of antigenic diversity inherent within this group is especially expressed within H and O somatic antigens. Ewing *et al.* (1961) described 12 O-antigen groups and 9 H-antigen groups. Each group was further divided into a number of additional serotypes. Chodyniecki (1965) also found a high degree of antigenic diversity among strains of motile aeromonads obtained from the same population of fish and even from different organs of the same fish. Monovalent bacterins were initially prepared against *A. hydrophila*, but these vaccines only provided acceptable levels of protection against challenge with a homologous bacterium. Fish were not immune to infection by heterologous strains of *A. hydrophila* (Post, 1966; Schaperclaus, 1967). Although some strains of motile aeromonads have common somatic antigens (Rao and Foster, 1977; Lallier *et al.*, 1981), it has been consistently demonstrated that a monovalent antiserum could agglutinate only a small percentage of the total isolates examined. Kingma (1978) produced seven rabbit antisera to heat stable antigens of seven isolates. Collectively, these antisera agglutinated only 19.5% of the total number of motile aeromonad isolates studied. Despite the tremendous degree of serological diversity that exists within this taxon, Mittal *et al.* (1980) found that strains of *A. hydrophila*, which were highly virulent for rainbow trout, shared a common O-antigen. Furthermore, these bacteria did not agglutinate in acriflavine and resisted the bactericidal activity of normal mammalian sera. In contrast, low-virulence strains of *A. sobria* did not share the common O-antigen; these bacteria settled after boiling and were lysed by normal mammalian sera. Schachte (1978) also found that the delivery of vaccine by injection or immersion or *per os* stimulated differential kinetics of antibody production. However, no significant protection against natural exposure to heterologous *A.*

hydrophila was afforded to channel catfish vaccinated by any of the methods. Anbarasu *et al.* (1998) found that formalin inactivated vaccines were superior to heat killed preparations with regards to protection against clinical disease, especially when the bacterins are injected with adjuvants. Thune and Plumb (1982c) found that both sac fry and swim-up fry vaccinated by immersion in sonicated polyvalent bacterin were protected against challenge with homologous bacteria, indicating an early onset of immunocompetence in channel catfish. This study was important because *A. hydrophila* causes a severe problem in channel catfish in spring and early summer, when fry are abundant. The early onset of immunocompetence among channel catfish fry indicates that immunization, if effective, could be used to reduce outbreaks of *A. hydrophila* infection. Regardless of whether whole cells, freeze-thawed cells, or cell sonicates were used, Thune and Plumb (1982c) further indicated that injection was superior to either immersion or spray vaccination for developing humoral antibodies. However, cell sonicates evoked the best antibody response. Sonication may disrupt the cell and allow better processing of certain somatic antigens (e.g. bacterial lipopolysaccharide). Consequently, Baba *et al.* (1988) were able to evoke better protection against A. hydrophila among carp that were vaccinated with crude lipopolysaccharide (LPS) rather than whole-cell, formalin killed vaccine. Immersion of fish in the LPS vaccine for 2 h at 25°C was more efficacious and less stressful than injection, but vaccination with crude LPS did not invoke an observed humoral immune response as measured by bacterial agglutination, passive haemagglutination and agar gel diffusion tests. Because of the antigenic diversity that exists among this complex group of organisms, additional vaccination strategies included research on development of polyvalent vaccines, immunization against inactivated extracellular toxins (toxoids), and the development of vaccines consisting of cellular antigens plus toxoid. Liu (1961) noted that the biological

activity of extracellular toxins from motile aeromonads was neutralized by a single antiserum prepared against an isolate of *A. liquefaciens* (synonym for *A. hydrophila).* He therefore concluded that the motile aeromonads shared extracellular antigens. Bullock *et al.* (1972) also indicated that aerogenic and anaerogenic strains of A. *hydrophila* possessed common extracellular antigens. Schaperclaus (1970) recognized that common carp *(Cyprinus carpio)* vaccinated against *A. hydrophila* developed both agglutinating serum antibodies against cellular antigens and antitoxic activity against extracellular antigens. In a later study, he found that common carp vaccinated by intraperitoneal injection of bacteria produced more circulating antibodies than did carp vaccinated by the oral route (Schaperclaus, 1972). Furthermore, vaccination with soluble extracellular antigens was more efficacious and provided a wider base of protection against heterologous serotypes than did vaccination with whole-cell antigen. Shieh (1987) found that Atlantic salmon immunized by intramuscular injections of extracellular protease from *A. hydrophila* were protected from challenge with the homologous and some heterologous isolates of *A. hydrophila*. Shieh (1990) repeated these studies and produced similar results when *A. sobria* extracellular protease was used as the immunogen and challenges were conducted with homologous and heterologous strains of the same bacterium. However, no report was made on the ability of extracellular protease derived from one of these species to cross protect against the other motile aeromonad species. Despite the great amount of research that has been expended to develop a safe and efficacious vaccine, there is still no product that has been licensed for use against the motile aeromonads within United States.

2.3.10 Epidemiology

Motile aeromonads cause diseases wherever bait fishes or warm water or ornamental fishes are propagated. To a lesser extent, these bacteria also initiate disease in cold-water species. Although diseases associated with motile aeromonads are most severe among fish that are propagated under conditions of intensive culture, these bacteria may also affect feral fish and are common in the intestinal flora of apparently healthy fish (Trust *et al.,* 1974). The bacterium is ubiquitous and occurs in most fresh water environments. It can be found both in the water column and in the top centimeter of sediment (Hazen, 1979). Motile aeromonads are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity, and temperature (Hazen *et al.,* 1978b*).* Temperature optimums may depend upon the particular strain under investigation, but generally range from 25°C to 35°C. Consequently, most epizootics among warm water fishes in the southeastern United States are generally reported in spring and early summer (Meyer, 1970). Pond water, diseased fish, and diseased frogs, as well as convalescent frogs and fishes, may become reservoirs of infection. Certain algae (Kawakani and Hashimoto 1978) and other protozoa (Chang and Huang, 1981) that are grazed upon by fish, can harbor motile aeromonads. In different study, *Tetrahymena pyriformis* was experimentally shown to graze on populations of *Aeromonas hydrophila.* The bacterium, at concentrations of $1x10^6$ cells/mL co-existed with the protozoan. If a second bacterial species was introduced, predation by the protozoan increased. In reservoir studies, Hazen (1979) found high densities of *Aeromonas hydrophila* in mats of decomposing *Mypiophyllum spiaatum* and, enterically, within largemouth bass, several other species of fish, turtles, alligators, and snails. The densities of *A. hydrophila* were highest in the water from March to June, although a second peak occurred in October. Largemouth bass from thermally altered parts of the reservoir had a significantly higher

incidence of infection. Kawakani and Hashimoto (1978) found greater quantities of *A. hydrophila* (as many as 10^3 cells) in algae fed upon by ayu (*Plecoglossus altivelis*) than were present in the water itself where the density of *A. hydrophila* was generally less than 10 cells/mL. Osborne *et al.* (1989) found high densities of motile aeromonads within the environment during midsummer when sedimentary chlorophyll and water temperature were highest. This also correlated temporally with the highest prevalence of dermally ulcerated striped mullet (Mugil cephalus) that also contain large concentrations of the bacteria within their stomachs and on their skin. The authors suggest that mullet graze on bacteria-laden sediment for algae and consequently bioaccumulate the pathogen within their guts and on their skin which, in turn, enhances disease. Thus, the intestinal tract or epidermal abrasions are likely portals of bacterial entry. Under conditions of stress, it is even likely that some strains of motile aeromonads that are ordinarily part of the normal gut flora become pathogenic. It is believed that infection occurs in winter, when fish are relatively inactive, and that the disease out breaks is commonly seen in spring. Aquarium fish, which are usually maintained at constant water temperature, can develop this disease at any time. Rainbow trout appear to be among the most susceptible of the salmonids that develop motile aeromonad septicemias (Cipriano *et al.,* 2001).

2.3.11 Zoonoses

Motile aeromonads can also cause disease in warm-blooded vertebrates. In immunocompromised human hosts, for example, *A. hydrophila* may cause septic arthritis, diarrhea, corneal ulcers, skin and wound infections, meningitis, and fulminating septicemias (von Gravenitz and Mensch, 1968; Davis *et al.,* 1978). Clinical isolates of *A. hydrophila* have been obtained from retail foods (fish, seafood, raw milk, poultry and red

meats) and all isolates had biotypes identical to those of enterotoxin-positive strains (Palumbo *et al.,* 1989). The ability of these bacteria to grow competitively at 5°C may be indicative of their potential as a public health hazard. However. Most *A. hydrophila* were reduced to non-detectable levels on catfish filets cooked to 70°C (Huang *et al.,* 1993).

Probably one of the more under appreciated routes by which *Aeromonas* wound infections can result is via bites of various animal species. The oropharyngeal flora of reptiles, and snakes in particular, often harbors aeromonads. Wound infections from cellulitis to necrotizing fasciitis have resulted from water moccasin, cobra, and viper snake bites (Angel *et al*., 2002). A stingray was found to cause a laceration in the dorsal aspect of the left foot of an 11-year-old boy swimming in a river in Argentina (Pollack *et al*., 1998). The resultant wound infection was edematous and tender with mild crepetation. The necrotic border of the lesion drained purulent fluid with a "fishy" smell. Wild animals such as grizzly bears (Kunimoto *et al.,* 2004) or tigers (Easow and Tuladhar, 2007) have been documented to produce traumatic bites on the scalp or shoulders, resulting in significant wounds containing aeromonads as part of mixed microbial populations. It is not clear in each of these instances what role, if any, aeromonads played in either the disease process or wound healing.

2.3.12 Methods of control

2.3.12.1 Prevention

Effective hatchery management is the best approach to avoid infections and subsequent epizootics caused by these bacteria. In water re-use hatcheries, both ozonation (Colberg and Lingg 1978) and filtration combined with ultraviolet irradiation (Bullock and Stuckey 1977) effectively eliminate the threat of *A. hydrophila*. Colberg and Lingg (1978) showed

that a specific microbial oxygen demand was exerted during batch ozonation that caused greater than a 99% mortality of bacteria within a 60-seconds contact during continuous flow exposure at 0.1-1.0 mg ozone per liter. Calesante *et al.,* (1981) eliminated recurrent motile aeromonad mortality among muskellunge (*Esox masquinongy*) fry by ultraviolet irradiation of lake and well water supplies during egg incubation and yolk absorption. Motile aeromonad septicemias are generally mediated by stress. Elevated water temperature (Esch and Hazen 1980), a decrease in dissolved oxygen concentration, or increases in ammonia and carbon dioxide concentrations have been shown to promote stress in fish and trigger motile aeromonad infections (Walters and Plumb 1980). The monitoring of environmental variables can therefore enable one to forecast stressful situations and possibly avoid problems before they arise. Wherever this disease occurs frequently, pond fish should not be handled but transferred only after water temperature is high enough for fish to be active and feeding normally (Rychlicki and Zarnecki 1957). Mortalities were reduced dramatically (80-90%) when fish, at the time of spring transfer, were injected intraperitoneally with 10-20 mg of chloromycetin or by dissolving 10-20 mg chloromycetin in water per kilogram of fish (5-10 mg/lb). In the United States, however, this drug is not registered for use on food fish nor is it legal to treat fish prophylactically (in the absence of disease). It is prudent that managers should avoid introducing fish into their hatcheries that have recently undergone infection. Shipments of new eggs should be surface disinfected to prevent contamination of facilities and stocks. Wright and Snow (1975) found that either acriflavine (500-700 mg/L for 15 min) or iodine as Betadine (100-150 mg/L iodine for 15 min) successfully disinfected eggs of largemouth bass *(Micropterus salmoides),* but neither Roccal nor formalin was effective. When warm water fishes are held in tanks or hauled in trucks or plastic bags, the value of adding disinfectants or

antibiotics should be examined. The most promising compounds include chloramphenicol, oxytetracycline, chlortetracycline, and a mixture of penicillin and streptomycin added to water at a rate of 10-15 mg/L. Again, it is important to note that the use and administration of such prophylaxis must be in compliance with local regulations.

2.3.12.2 Treatment

Oxytetracycline (Terramycin) has been the drug of choice for treating motile aeromonad septicemias in fishes. The drug is approved for use with pond fishes, channel catfish, and salmonids. It is administered in feed at a daily rate of 50 to 75 mg/kg of fish for 10 days. Fish must be withdrawn from treatment for 21 days before they are smocked or eaten. This treatment sometimes produces dramatic results when it is administered for even 2 or 3 days, and is particularly effective when fish become infected after they have been handled, crowded, or held under stress for short periods of time (Meyer 1964; Meyer and Collar 1964). Chloromycetin®, like oxytetracycline, was effective in treating fish when it was administered orally. However, its use is prohibited in food fishes and discouraged in other fishes because it is the drug of last resort in certain human diseases e.g., typhoid fever. Indiscriminate use of chloramphenicol can result in drug resistance and thus reduce the value of the antibiotic in human medicine. Against strains of motile aeromonads that show multiple drug resistance, piromidic acid administered orally has been experimentally shown to be more effective than either chloromycetin or oxytetracycline (Katae *et al.,* 1979).

2.3.13 Antimicrobial susceptibilty

The susceptibility status of *Aeromonas* isolates for therapeutically active drugs also appears to be independent of species designation. Such a conclusion takes into consideration that most *A. trota* strains are susceptible to ampicillin yet use of this ß-lactam is contraindicated

in regards to treatment of *Aeromonas* infections. While some species-specific susceptibility differences have been found in some selected studies, such results should be considered preliminary (Kampfer *et al*., 1999; Overman and Janda, 1999). There were no significant differences in the susceptibilities of aeromonads to antimicrobial agents based upon origin of isolation (clinical versus environmental), although certainly more studies need to be performed in this area (Kampfer *et al*., 1999). The general susceptibility profile of the genus *Aeromonas* for class-specific antibiotics is depicted in Table 2.2. However, this table should be viewed only as a general baseline for the genus, given that percentages of drug resistance may vary significantly due to individual species, geographic locales, or environmental selection pressures.

Table 2.2: General susceptibility profiles for most clinically relevant *Aeromonas* isolates.

Susceptibility profile (% of isolates)[j]	Antibiotic family
Susceptible (90–100) ----------	Aminoglycosides
	Carbapenems
	Cephalosporins (extended spectrum)
	Cephalosporins ("fourth generation")
	Macrolides[g]
	Monobactams
	Nitrofurans
	Penicillins (extended spectrum) [i]
	Phenicols
	Quinolones
	Tetracyclines
Variable (70–90) ----------	Aminoglycosides[a]
	Antifolates[c]
	Cephalosporins[d] (expanded spectrum)
Resistant (70) ----------	Antifolates[b]
	Cephalosporins (narrow spectrum)
	Penicillins[e] (extended spectrum)
	Macrolides[f]
	Penicillins[h] (narrow spectrum)

[a] Tobramycin.
[b] Sulfamethoxazole.
[c] Trimethoprim-sulfamethoxazole.
[d] Cefoxitin.
[e] Amoxicillin, ampicillin, ampicillin-sulbactam, ticarcillin.
[f] Clarithromycin.
[g] Azithromycin.
[h] Oxacillin, penicillin.
[i] Azlocillin, piperacillin, piperacillin-tazobactam.
[j] Percentages of susceptible isolates were derived from references (Kamper *et al*., 1999; Overman and Janda, 1999; Janda, 2001; Zhiyong *et al.*, 2002)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Identification of Mango Kernel

Mangifera indica kernel were collected from mango trees around Ahmadu Bello University (ABU) Samaru, Zaria. The leaves were identified at Herbarium Department of Biological Sciences, A.B.U. Zaria, where a voucher specimen no. 1944 was assigned. The kernel was washed repeatedly under running tap water. It was air-dried at room temperature and then made into powder using pestle and mortar. The powder was preserved in an airtight polythene bag and kept in a cool dry place until used.

3.2 Extraction

3.2.1 Preparation of the aqueous extract of mango kernel

Concentrations of 50, 55.6, 100, 125, 166, 250 and 500 mg/ml of the powdered mango kernel were prepared using sterile distilled water. To 1g of the grounded kernel 20ml of distilled water was added to achieved 50mg/ml concentration, in a similar manner; 1g in18ml, 1g in12ml, 1g in10ml, 1g in 8ml, 1g in 6ml, 1g in 4ml and 1g in 2ml for 55.6, 100, 125, 166, 250,500 mg/ml concentrations respectively. The different concentrations of the extract were refrigerated at 4˚C for 24 hours before use.

3.2.2 Preparation of methanol extract of mango kernel

powdered material (200g) was extracted exhaustively over the period of 24 hours using continuous extraction method with 350ml of methanol in a soxhlet extractor at the New Pharmacognosy Laboratory, Faculty of Pharmaceutical Sciences, A.B.U. Zaria. The extract

was concentrated to solid form using water bath at 60°. To 1g of the solidified methanol extract 20ml of distilled water was added to achieved 50mg/ml concentration, in a similar manner; 1g in18ml, 1g in12ml, 1g in10ml, 1g in 8ml, 1g in 6ml, 1g in 4ml and 1g in 2ml for 55.6, 100, 125, 166, 250,500 mg/ml concentrations respectively. The different concentrations of the methanol extract of mango kernel were refrigerated at 4˚C for 24 hours before use.

3.2.3 Phytochemical screening

Phytochemical screening of the methanol extract of mango kernel was carried out to identify the constituents, using standard phytochemical methods as described by Evans (2009). The screening involves detection for presence or absence of alkaloids, flavonoids, steroids, terpenoids, saponins, tannins, anthraquinones, cardiac glycosides and reducing sugars.

3.2.4 Test for carbohydrates:

3.2.4.1 Molisch test

Three drops of Molisch reagent was added to 2ml of the methanol extract in a test tube, 1 ml of concentrated sulphuric acid was added to the mixture down the side of the test tube.

***3.2.4.2** Fehling test*

To 1 ml of the methanol extract in a test tube, 5ml of an equal volume of mixture of Fehling solution A and B was added and boiled on a water bath.

3.2.5 Test for anthraquinones:

3.2.5.1 Test for free anthracene derivatives (Bontrager's test)

To 1 ml of the methanol extract in a test tube, 5 ml of chloroform was added and was shaken for 5minutes. It was filtered and the filtrate shaken with equal volume of 10% ammonia solution.

3.2.5.2 Test for Combined Anthracene Derivatives (Modified Bontrager's Test)

Small portion (1 ml) of the methanol extract in a test tube was boiled with 5 ml of 10% hydrochloric acid for 2- 3 mins. This hydrolysed the glycosides and yield aglycones that are soluble in hot water. This was then filtered and the filtrate was cooled and extracted with 5 ml of chloroform. The benzene layer was pipetted off and shaken gently in a test tube with half of its volume of 10% ammonium hydroxide.

3.2.6 Test for unsaturated steroid and triterpene:

3.2.6.1 Liehermann-Bucchard Test

To 1 ml of the methanol extract, equal volume of acetic acid anhydride was added and mixed gently. 1 ml of concentrated sulphuric acid was added down the side of the test tube which forms a lower layer.

3.2.6.2 Salkowski test for unsaturated sterols

To 1 ml of the methanol extract, three drops of concentrated sulphuric acid was added at the side of the test tube. The mixture was left in the test tube over a period of 1 hour for observation.

3.2.7 Test for cardiac glycoside:

3.2.7.1 Keller- Kiliani Test

Two (2) ml of the methanol extract was dissolved in 1 ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1 ml of concentrated sulphuric acid was added down the side of the test tube which formed a lower layer at the bottom.

3.2.7.2 Kedde's Test

To 1 ml of the methanol extract, 1 ml of 2% solution of 3,5-Dinitrobenzoic acid in 95% alcohol was added. The solution was made alkaline by addition of 5% sodium hydroxide.

3.2.8 Test for saponins:

3.2.8.1 Frothing

Ten (10) ml of distilled water was added to 4 ml of the methanol extract and was shaken vigorously for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30 minutes.

3.2.9 Test for tannins:

3.2.9.1 Ferric Chloride Test

To 1 ml of the methanol extract, three drops of ferric chloride solution was added and observed over the period of 5 minutes.

3.2.9.2 Lead Sub-acetate Test

To a small portion (1) ml of the methanol extract, three drops of lead sub-acetate solution was added which gives a precipitate.

3.2.10 Test for flavonoids:

3.2.10.1 Shinoda Test

Two (2) ml of the methanol extract was dissolved in 1 ml of 50% methanol in the heat metallic magnesium chips and few drops of concentrated hydrochloric acid were added.

3.2.10.2 Sodium Hydroxide Test

Three drops of 10% sodium hydroxide was added to 1 ml of the methanol extract in a test tube.

3.2.11 Test for alkaloids:

3.2.11.1 Mayer's Test

To 1 ml of the methanol extract, three drops of Mayer's reagent was added and observed over the period of 5 minutes for any reaction or colour change.

3.3 Acute Toxicity Test

3.3.1 Limit test

Fish acute toxicity test was carried out as described in OECD (Organisation for Economic Cooperation and Development) testing protocol 203 (1992) with modification. Catfish fingerlings (n= 60, length 10.5 ±1.0 cm, weigth 10 ± 1.0g) were used for the study, they were divided randomly into six (6) groups of 10 fingerlings (4 weeks old). The fish were obtained and held in the laboratory for 12 days before they were used for the test. They were held in water of the quality to be used in the test for seven days (in semi static tanks) immediately before testing and under conditions recommended by OECD (1992). Mango kernel powder was added into an experimental feed at different doses (0, 1, 4, 20, 40, 60 g/kg of feed). The feeds were given to different fish treatment groups twice daily for 96

hours (4 day). Fish were observed at 24, 48, 72 and 96 hours for any mortality or clinical signs.

3.3.2 Water quality test

Water quality characteristics; pH values, temperature (°C), general hardness (ppm mg/l), carbonate hardness (ppm mg/l), nitrite (NO^{-}_{2} ppm mg/l), nitrates (NO^{-}_{3} ppm mg/l) of the water in different tanks were determined using a test kit (API^{TM} Aquarium Pharmaceuticals Mars Fish care North America, inc.).

Table 3.1: Water quality test

Treatment with M.indica kernel in g/kg of feed	Days (hours)	pH	NO_2 ppm (mg/l)	NO^-_3 ppm (mg/l)	Temp. °C	Carbonate hardness (CH) ppm (mg/l)	General hardness (GH) ppm (mg/l)
0	24	7.0	0	0	24	80	30
	48	7.0	0	0	24	80	30
	72	7.0	0	0	24	80	30
	96	7.0	0	0	26	80	30
1	24	7.0	0	0	24	80	30
	48	7.0	0	0	24	80	30
	72	7.0	0	0	24	80	30
	96	6.5	0	0	25	80	30
4	24	7.0	0	0	24	80	30
	48	7.0	0	0	24	80	30
	72	7.0	0	0	24	80	30
	96	7.0	0	0	26	80	30

Table 3.2: Water quality test

Treatment with M.indica kernel in g/kg of feed	Days (hours)	pH	NO_2 ppm (mg/l)	NO^-_3 ppm (mg/l)	Temp. °C	Carbonate hardness (CH) ppm (mg/l)	General hardness (GH) ppm (mg/l)
20	24	7.0	0	0	24	80	30
	48	7.0	0	0	24	80	30
	72	7.0	0	0	24	80	30
	96	7.0	0	0	26	80	30
40	24	7.0	0	0	24	80	30
	48	7.0	0	0	24	80	30
	72	7.0	0	0	24	80	30
	96	7.0	0	0	26	80	30
60	24	7.0	0	0	23	80	30
	48	7.5	0	0	24	80	30
	72	7.0	0	0	24	80	30
	96	6.5	0	0	27	80	30

3.4 Feeding Diet and Grouping of Fish

Catfish fingerlings (n= 240) were used for the study, they were divided randomly into 4 groups of 60 fingerlings (4 weeks old). Each group was maintained in a 500 litre tank. Fish in group A were fed basal diet and served as non- treated control group, while fish in groups B, C and D were given feed containing mango kernel at 1, 5 and 10 g/kg of feed (Table: 3.3).

3.5 Preparation of the Herbal Diet

Three experimental diets (fish diet) were prepared by incorporating mango kernel powder at 1, 5 and 10 g/kg of feeds (Table 3.3). The powdered mango kernel was mixed thoroughly with other ingredients (Table 3.3) and a binder (1%), three hundred (300) ml of water was added to achieve the required consistency. The resulting dough was pelleted using a locally made sieve, the pellets were dried at room temperature for 48 hour and then stored in airtight containers, which served as different experimental fish diet. The mango kernel free feed (basal diet) was used as a control diet for fish in Group A.

Table 3.3: Composition of feed fed to different experimental catfish fingerlings.

Ingredients	Group A (Untreated control)	Group B	Group C	Group D
Groundnut cake (g)	400	400	400	400
Fish meal (g)	250	250	250	250
Rice bran (g)	200	200	200	200
Soyabean meal (g)	120	120	120	120
Vitamin and mineral mixture (g)	20	20	20	20
Starch (g)	10	10	10	10
Mango kernel powder (g)	0	1	5	10

3.6 Collection of Blood and Serum

Blood samples were collected randomly from 12 fish in each group 20 and 40 days post-treatment. 0.5 ml of blood was collected from the caudal vein of each fish using a caudal vein severance and through the vein using a 23 gauge needle attached to a 2 ml syringe respectively. The blood was analysed for packed cell volume, Hb and WBC counts.

To obtain serum, another different 12 fish from each group were used. 0.5 ml of blood was collected from each fish and into anticoagulant free 5ml bottles and allowed to stand for 2 hours to clot. Thereafter the clotted blood was centrifuged at 400 g for 5 minutes and the serum was kept at -8˚C until used.

3.7 Antimicrobial Activity Test

The agar well diffusion method (Perez *et al*., 1990; Alade and Irobi, 1993; Abioye *et al*., 2004) was used to test for antimicrobial activity of the serum, methanol and Aqueous extracts of mango kernel. Overnight culture of *A. caviae* grown on Mueller–Hinton agar plates were used. Colony of cultured bacteria was applied onto sterile plate containing Mueller–Hinton agar. Five wells (6 mm in diameter) were made equidistance in each of the plates using a sterile cup borer. One hundred microlitres of different concentrations of both water and methanolic extracts of mango kernel were put individually into the wells using sterile pipettes and 100 µl of sterile distilled water in a given well. Thereafter, the plates were incubated at 37˚C for 24 hours. It was allowed to diffuse at room temperature for 2 hours. The plates were incubated at 37^{o}C for 24 hours. The diameter of the inhibition zones (mm) were measured after the 24 hours of incubation using centimeter rule.

The Agar Disc Diffusion (Kirby-Bauer method) which conforms to the recommendation of NCCLS was used to evaluate the activity of standard antibiotics (Bauer *et al.,* 1966) (NCCLS, 1998). Six different antibiotics Gentamicin (G) 10 µg, Enrofloxacin (EN) 5 µg, Erythromycin (E) 5 µg, Neomycin (N) 10 µg, Chloramphenicol (C) 30 µg and Tetracycline (T) 30 µg were gently placed on Mueller–Hinton agar plate inoculated with *A. caviae*. The plate was incubated at 37^{o}C for 24 hours. The diameter of the inhibition zones (mm) were measured after the 24 hours of incubation using centimeter rule.

Two sets of five different serum samples collected after the first 20 and 40 days of feeding from all the four groups of fish were also introduced into the wells on Mueller–Hinton agar using sterile pipettes. The sera were also allowed to diffuse into the agar plate at room temperature for 2 hours, thereafter incubated at 37^{o}C for 24 hours. The diameter of the inhibition zones (mm) were measured after the 24 hours of incubation using centimeter rule.

3.8 Prophylactic Trials

Twelve fish from each group were randomly sampled and challenged through interperitoneal route with 6.7 x 10^{11} cfu/ml of *A. caviae* (sourced from the Department of Veterinary Microbiology, A.B.U, Zaria). Clinical signs and mortality were observed and recorded, Gross lesions in the dead fish were also observed and recorded based on different groups that they were assigned for the period of two (2) weeks.

3.9 Isolation of *A. caviae* from Infected Fish

A. caviae was isolated from the fish that died in the untreated group (group A) and group treated with mango kernel at 1 g/kg of feed (group B), the isolation was carried out in the

Department of Veterinary Microbiology, Ahmadu Bello University, Zaria. Liver from the dead fish were removed and laid on a clean aluminium foil sheet, the liver was aseptically sneared using a sterile spatula, lanced then using a sterile swab stick it was gently inoculated onto a blood agar supplemented with 5% defibrinated sheep blood and grown in an incubator at 32°C for 24 hours. The presence of gray, raised and moist colonies with zones of hemolysis around were observed, which indicated Aeromonas organisms. Furthermore, the organism was subcultured on Tryptic soy agar and incubated at 32°C for 24 hours, then subjected to further microscopic examination and biochemical test.

3.9.1 Microscopic identification of *A. caviae*

Colonies of the organism observed on Tryptic soy agar were Gram stained, and observed under x100 magnification using compound microscope. Isolates that were rod shaped, Gram negative cells under microscope were considered *Aeromonas*.

3.9.2 Biochemical identification of *A. caviae*

Presence of the enzyme cytochrome oxidase was observed using oxidase test strip. The strips were impregnated with a colourless redox dye (tetramethyl-p-phenylenediamine dihydrochloride) and ascorbic acid (MICROBACT Oxidase Strip MBO 266 A OXOID RG24 8PW, UK). Catalase test, Voges proskauer, Motility, Indole and Urea test were all conducted. Fermentation of the following sugars was tested: Production of gas from glucose, sucrose, lactose, mannose, arabinose, rhamnose, sorbitol and dositol.

3.10 Determination of Blood Parameters

Packed Cell Volume (PCV) was analysed by centrifuging blood samples collected from fish in all the 4 groups in a capillary tube at 400 g for 5 minutes. Haemoglobin content

was analysed using cyanomethemoglobin method as described by Van Kampen and Zijlstra (1961). WBC count was determined by modified leishmann staining technique as described by Cheesbrough (2000) with slight modification. Briefly the slides were covered with undiluted stain, and diluted to twice its volume with water and allowed to stand for 10 minutes. Excess stain was washed off with tap water, air dried and observed microscopically under oil immersion. One hundred leucocytes (neutrophils, lymphocytes, monocytes, basophils and eosinophils) were counted on each slide. Total leucocytes counts were calculated using the formula below as described by Campbell (1995).

$$\text{TWBC/mm}^3 = \frac{\text{number of cells counted} \times 10 \times 32 \times 100}{(\%\text{H+E}) \times 18}$$

3.11 Data Analysis

Descriptive statistics was used to measure the percentage survivability between control and the treatment groups. Student t-test was also used to identify any statistical difference between groups. Data obtained from hematological parameters are expressed as mean ± S.E.M and analysed using one-way analysis of variance (ANOVA). Differences between means from different groups was analysed using Tukey's Post-hoc test. Statistical differences was considered significant at $p \leq 0.05$. All data were analysed using prism 5 computer software (Graphpad prism Version 5.01,San Diego, USA).

CHAPTER FOUR

RESULT

4.1 Phytochemical Constituent of *M. indica* kernel

The qualitative phytochemical screening test revealed presence of carbohydrates, triterpenes, unsaturated sterols, cardiac glycosides, saponin, condensed tannins, hydrolysable tannins and flavonoids (Table:4.1).

Table 4.1: Phytochemical Constituent of Mango kernel.

Phytochemicals	inference
Carbohydrates	+
Anthraquinones	-
Triterpenes	+
Unsaturated sterols	+
Cardiac glycosides	+
Saponin	+
Tannins	+
Flavonoids	+
Alkaloids	-

+ = Present
- = Absent

4.2 Median Lethal Dose (LD_{50}) of *M. indica* kernel Powder

4.2.1 Limit Test

The result obtained following the test procedure demonstrates that the lethal dose (LD_{50}) of *M. indica* kernel powder is greater than 60 g/kg of feed (Table 4.2).

Table 4.2: Safety of *M. indica* kernel powder at different doses in *C. gariepinus* fingerlings (10 fingerlings per treatment group).

Treatment with *M.indica* kernel (g/kg of feed)	Mortality in different treatment groups (hours)			
	24	48	72	96
0	-	-	-	-
1	-	-	-	-
4	-	-	-	-
20	-	-	-	-
40	-	-	-	-
60	-	-	-	-

- = Absence of mortality

4.3 Serum Antimicrobial Activity

The serum antimicrobial activity of fish fed diet containing mango kernel at 0, 1, 5 and 10 g/kg of feed, on days 20 and 40 post-treatment has not showed any antimicrobial activity against the test organism (*A. caviae*) (Table 4.3). Plates (I, II, III and IV) showed serum seeded into the wells of Muellar-Hinton agar (Modified agar well diffusion method) with no observable inhibition of the *A. caviae* in the media.

Table 4.3: Serum antimicrobial activity of fish fed diet incooperated with *M.indica* kernel at different doses, using modified agar well diffusion method.

Treatment with *M. indica* kernel g / kg of feed	Amount of serum/ well in µl	Inhibition zones (mm)	
		20 days post-treatment	40 days post- treatment
0	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
1	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
5	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
10	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI
	100	NI	NI

NI = No observable inhibition zone.



Plate I: Agar well diffusion test of serum of fish fed non-treated diet for 40 days.

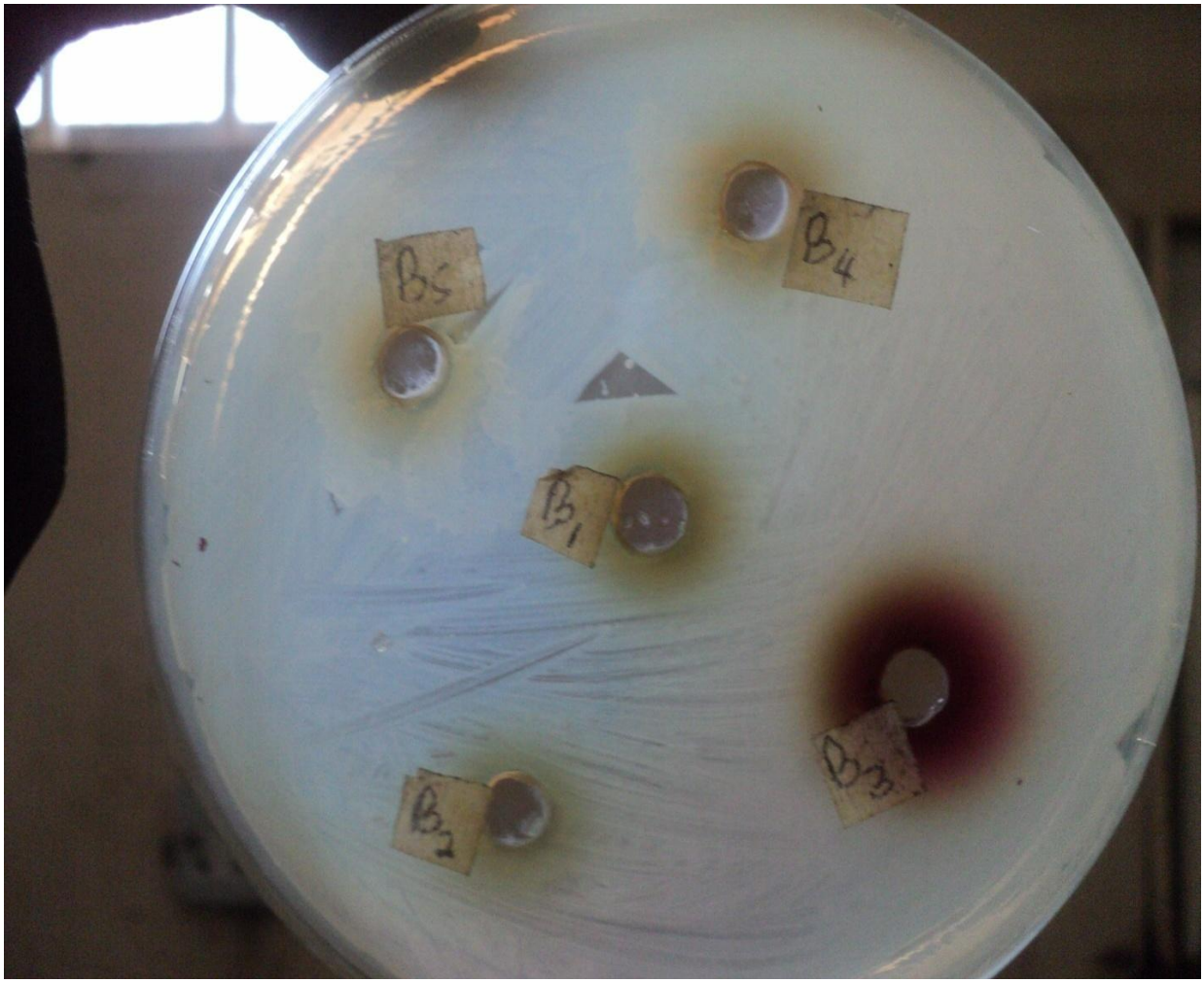


Plate II: Agar well diffusion test of serum obtained from fish fed diet containing mango kernel at 1 g/kg of feed for 40 days. No discernible zone of antimicrobial effect was observed.

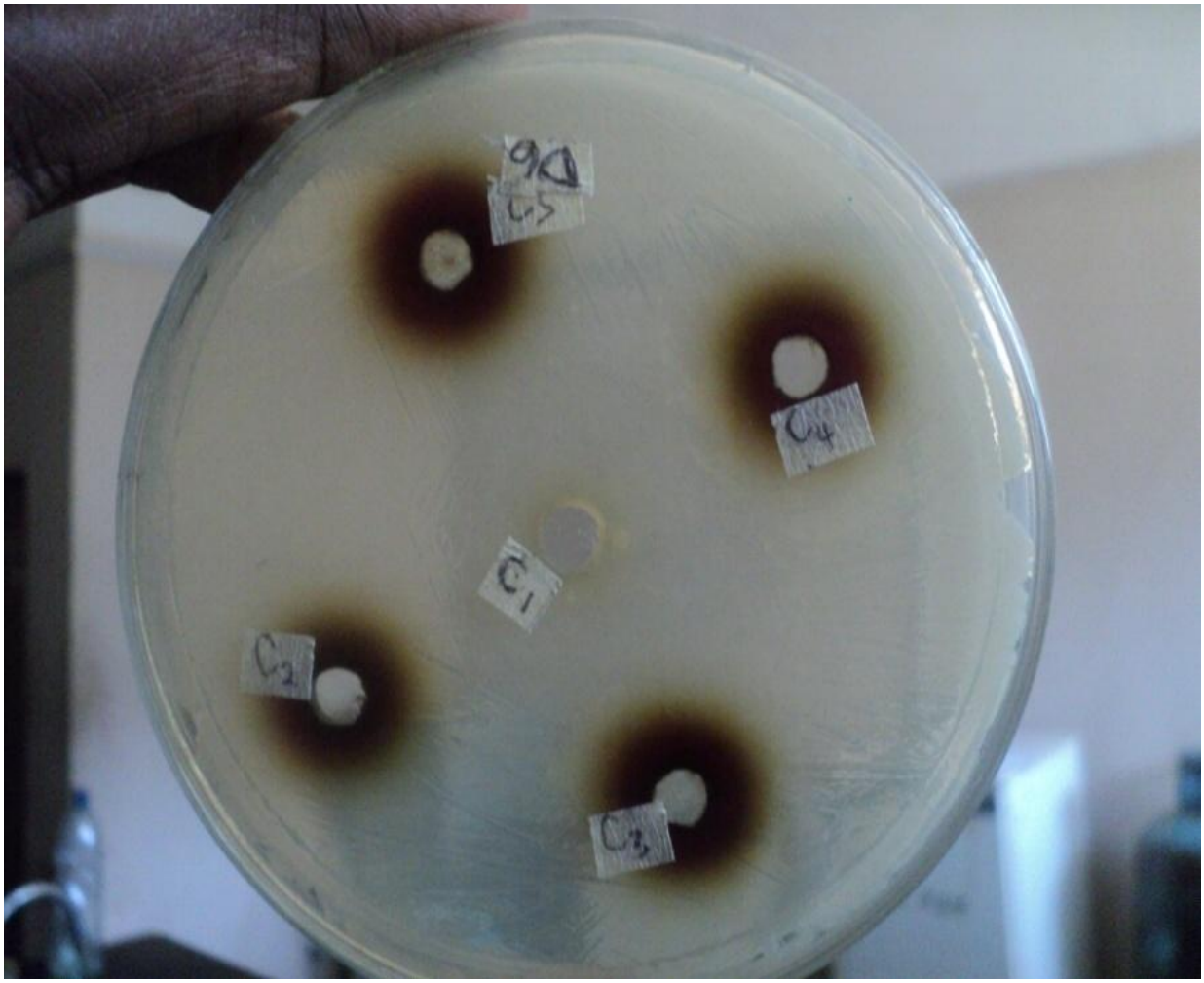


Plate III: Agar well diffusion test of serum obtained from fish fed diet containing mango kernel at 5 g/kg of feed for 40 days. No discernible zone of antimicrobial effect was observed.

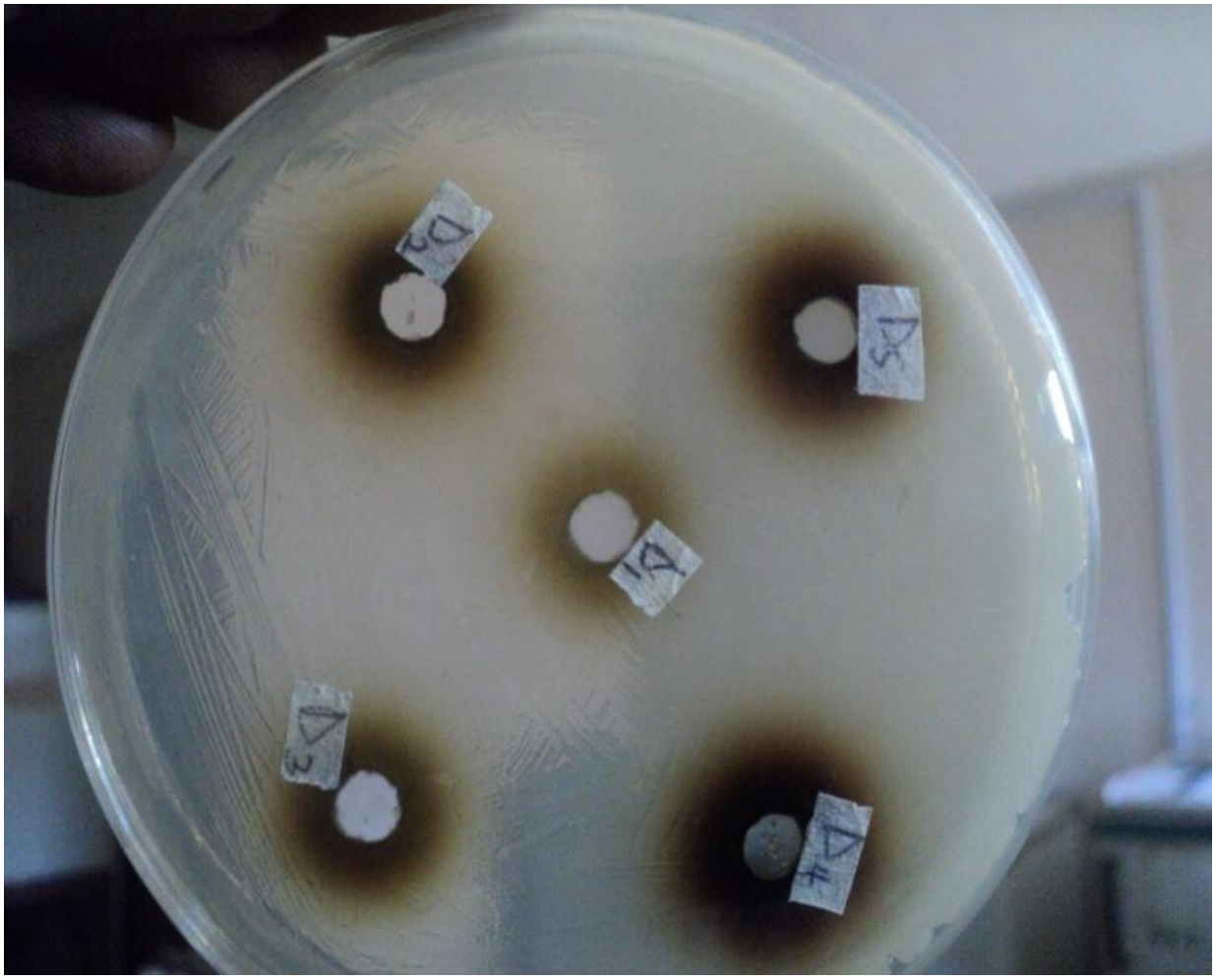


Plate IV: Agar well diffusion test of serum obtained from fish fed diet containing mango kernel at 10 g/kg of feed for 40 days. No discernible zone of antimicrobial effect was observed**.**

4.4 Antimicrobial Activity Test of *M. indica* kernel Methanol Extract, Aqueous Extract and Standard Antimicrobials

Methanol extracts of *Mangifera indica* kernel was evaluated for *in vitro* antimicrobial potential by agar well diffusion assay (Table 4.4) Almost all the methanol extracts used at different concentrations showed varying degrees of inhibition against the bacterial specie (*A. caviae*). Methanol extract was found to be comparatively more effective with inhibition zone sizes ranging from 16 mm to 24 mm. The methanol extracts showed inhibition zones of 16 ± 2.41, 17 ± 2.19, 18 ± 1.53, 20 ± 0.33, 20 ± 0.33, 23 ± 0.33 and 24 ± 0.58 mm diameter range at concentrations of 50, 55.6, 100, 125, 166, 250 and 500 mg/ml respectively, with a distilled water in a given well showing no inhibition (Plate V). However, aqueous extract was found to be slightly active against the test organism at lower concentrations with zones of inhibition ranging from 8 ± 1.00 to 11 ± 1.00, but there were no measurable zones of inhibition at higher concentrations (Table 4.5). Standard antibiotics showed degrees of inhibition in the range of 14 mm to 20 mm, slightly lower than that of methanol extracts at the concentrations used, but tetracycline has not showed measurable inhibition zones. The standard antibiotics showed variable zones of inhibition of 20 mm (G), 18 mm (EN), 17 mm (E), 15mm (N), 14mm (C), and 0 mm (T) that is Tetracycline (Plate VI and Table 4.6).

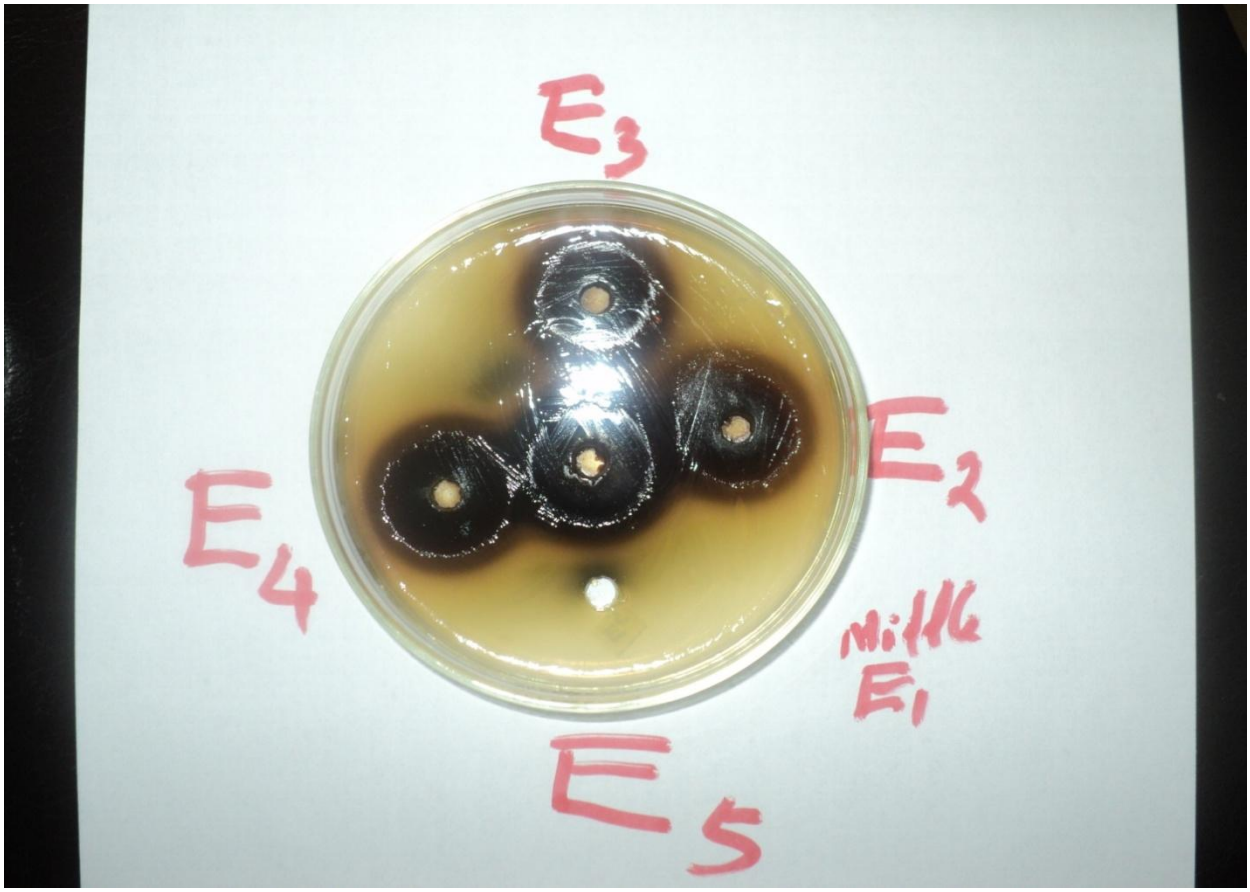


Plate V: Zones of inhibition at four (4) different concentrations (125, 166, 250 and 500 mg/ml) of the methanolic extract of *M. indica* kernel, E_1 – E_4 respectively with well E_5 (100µl of distilled water) showing no inhibition.

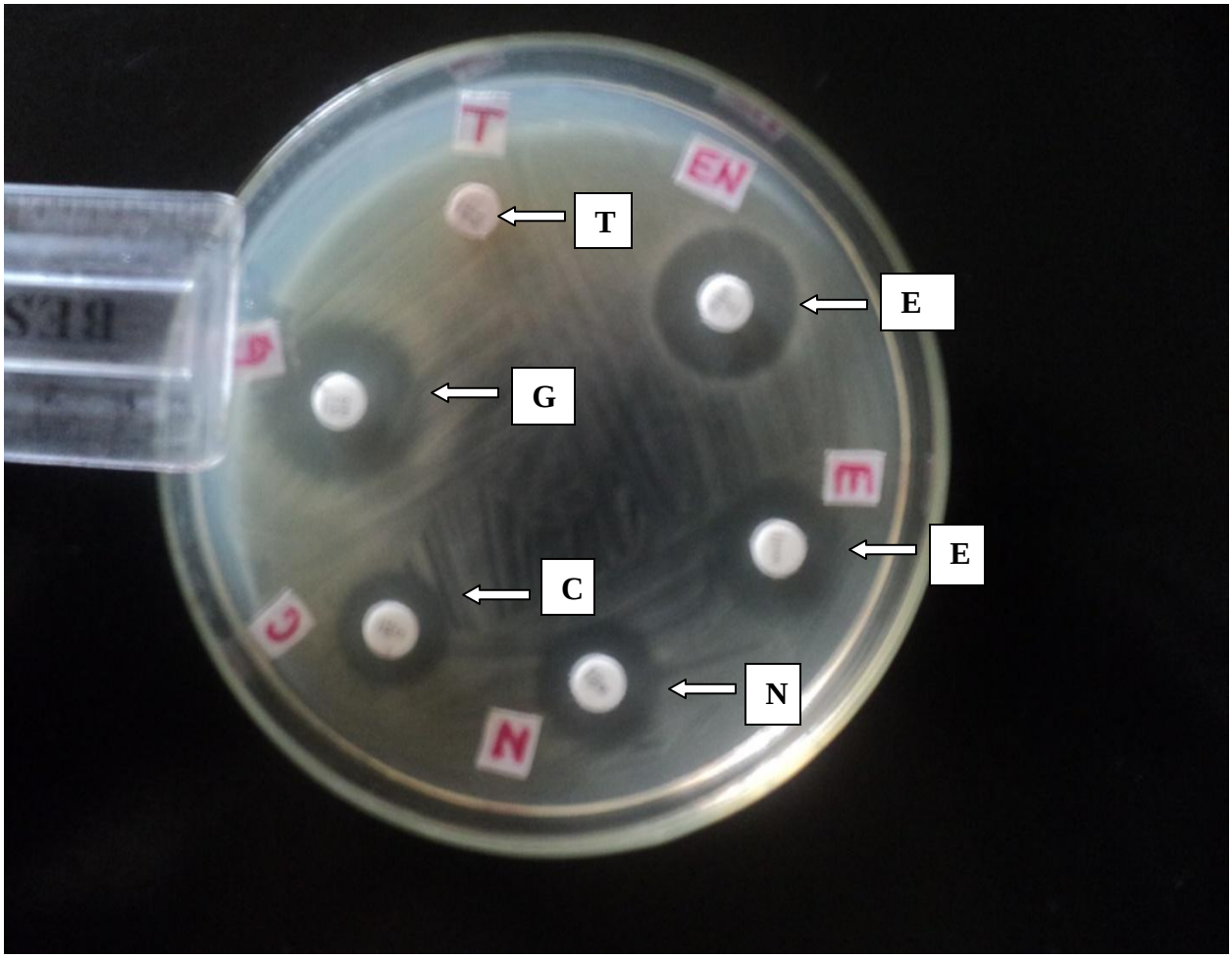


Plate VI: Different antibiotics at different concentrations showing variable degree of inhibition, Gentamicin 10 µg (G), Enrofloxacin 5 µg (EN), Erythromycin 5 µg (E), Neomycin 10 µg (N), Chloramphenicol 30 µg (C) and Tetracycline 30 µg (T) (Agar disc diffusion method).

Table 4.4: ***In vitro*** **antimicrobial activity of methanol extracts of** ***M. indica*** **kernel on** ***A. caviae*** **using modified agar well diffusion method.**

Concentration (mg / ml)	Diameter of inhibition zones (mm)
50	16 ± 2.41
55.6	17 ± 2.19
100	18 ± 1.53
125	20 ± 0.33
166	20 ± 0.33
250	23 ± 0.33
500	24 ± 0.58

Results are presented as Mean ± SD

Table 4.5: *In vitro* antimicrobial activity of aqueous extract on *A. caviae* using modified agar well diffusion method.

Concentration (mg / ml)	Diameter of inhibition zones (mm)
50	8 ± 1.00
55.6	9 ± 0.50
100	9 ± 1.50
125	8 ± 0.50
166	11 ± 1.00
250	NI
500	NI

NI = No observable inhibition (Note: Original well size is 6mm diameter), Results are presented as Mean ± SD.

Table 4.6: *In vitro* antibacterial activity of six (6) different antibiotics on *A. caviae* using agar disc diffusion method.

Standard Antibiotics	Conc. (mg)	Diameter of inhibition in mm
Chloramphenicol (30µg)	0.03	14
Enrofloxacin (5µg)	0.005	18
Erythromycin (5µg)	0.005	17
Gentamycin (10µg)	0.01	20
Neomycin (10µg)	0.01	15
Tetracycline (30µg)	0.03	-

4.5 Prophylactic Trials

Mortalities were recorded in fish from untreated group (group A) and group treated with mango kernel at 1 g/kg of feed (group B). Sluggishness, erratic swimming and isolation were noticed in groups A and B. Fish fed both treated and untreated diet (at all doses) had reduced feed intake (Table 4.7). Two (2) and one (1) fish died in groups A and B respectively, one day after infection with *A. caviae*. Similarly, two (2) days post-infection, two (2) fish died in group A. finally on the fifth day two (2) and one (1) fish died in groups A and B respectively. Therefore, the total number of fish that died in groups A and B were 6 and 2 respectively.

Post-mortem finding in fish that died revealed exopthalmus, discoloured skin with areas of slight erosion, pale liver and kidney and distended abdomen (Table 4.8) (Plates: VII) and there were no mortalities from day six (6) through day fourtheen (14) post challenge (Table 4.9).

Table 4.7: Clinical signs recorded from both treated and untreated groups after challenge with *A. caviae.*

Clinical manifestation	Treatment with *M.indica* at g/kg of feed			
	0	1	5	10
Sluggishness	+	+	-	-
Skin discolouration	+	+	-	-
Erratic swimming	+	+	-	-
Floating on the surface of the pond	+	+	-	-
Reduced feed intake	+	+	+	+

(+) = Present
(-) = Absent

Table 4.8: Gross lesions observed in the fish that died from both treated and untreated groups after challenge with *A. caviae.*

Gross lesions	Treatment with *M.indica* at g / kg of feed	
	0	1
Discolouration of the liver	+	+
Exopthalmus	+	-
Distended abdomen	+	+
Discolouration of the skin	+	+
Ulceration / erosion	+	-

(+) = Present
(-) = Absent

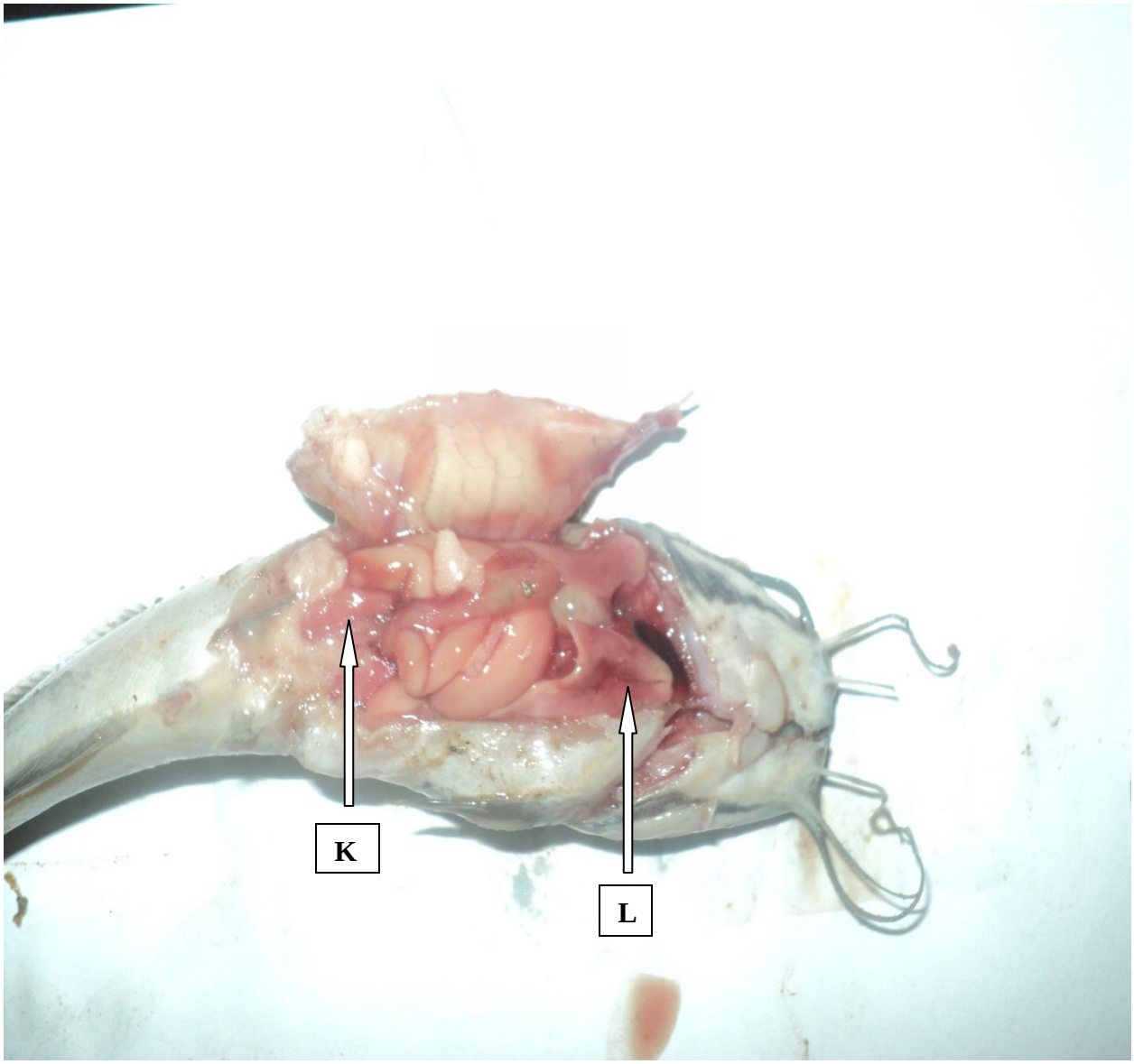


Plate VII: Gross pathologic lesion in fish fed with untreated diet note the discoloured liver (L) and kidney (K).

Table 4.9: Effect of *Mangifera indica* kernel on the survival of *Clarias gariefinus* challenged with *A. caviae* (6.7×10^{11} cfu / ml) (12 fish per group).

Treatment with *M. indica* kernel at g / kg of feed	Survival (%) and number of fish survived (in each group) time after challenged (in days)				
	1	2	3	4	5
0	83.33 (10)	66.66 (8)	66.66 (8)	66.66 (8)	50 (6)
1	91.66 (11)	91.66 (11)	91.66 (11)	91.66 (11)	83.33 (10)
5	100 (12)	100 (12)	100 (12)	100 (12)	100 (12)
10	100 (12)	100 (12)	100 (12)	100 (12)	100 (12)

Values in the parenthesis indicates number of fish survived.

4.6 Isolation of *A. caviae* from Infected Fish

Gram negative rods were identified under microscope, they were subjected to biochemical test and subsequently sugar fermentation test and results obtained are as in the following Tables (Table 4.10 and 4.11).

Table 4.10: Results of Oxidase and Catalase test and other biochemical test on *A. caviae.*

Test	Inference
Catalase	+
Oxidase	+
Indole	+
Urease	-
Voges Proskauer	-
Motility	+

(+) = Present

(-) = Absent

Table 4.11: Results of Sugar fermentation test.

Test	Inference
Production of gas from glucose	+
Sucrose	+
Lactose	-
Mannose	+
Arabinose	+
Rhamnose	+
Sorbitol	+
Glucose	+

(+) = Present

(-) = Absent

4.7 Hematological Parameters

Haematological parameters (PCV, Hb and WBC) of African catfish fed different doses of *M. indica* kernel for 20 and 40 days respectively (Tables 4.12 and 4.13).

Table 4.12: Mean ± SEM haematological values of African catfish 20 days after being fed with different doses of *M. indica* kernel (n =12).

Treatment with mango kernel at g / kg	PCV (%)	Hb (g / dl)	TP (%)	TLC ($x10^3/mm^3$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
0	31.00 ± 3.35	10.30 ± 1.12	5.04 ± 0.31	1.87 ± 0.02	5.20 ± 1.36	94.40 ± 1.29	0.00 ± 0.36	0.40 ± 0.25	0.00 ± 0.14
1	35.40 ± 3.08	11.78 ± 1.02	5.80 ± 1.39	1.88 ± 0.02	5.60 ± 1.03	94.40 ± 1.03	0.00 ± 0.26	0.00 ± 0.37	0.00 ± 0.28
5	37.20 ± 1.86	12.36 ± 0.61	6.84 ± 0.52	1.88 ± 0.02	5.80 ± 0.80	93.40 ± 0.51	0.60 ± 0.40	0.60 ± 0.40	0.00 ± 0.39
10	39.00 ± 1.52	12.96 ± 0.51	6.86 ± 0.45	1.93 ± 0.01	8.40 ± 0.40	91.20 ± 0.49	0.40 ± 0.25	0.40 ± 0.25	0.00 ± 0.18

Table 4.13: Mean ± SEM., haematological values of African catfish 40 days after being fed with different doses of *M. indica* kernel (n =12).

Treatment with mango kernel at g/kg	PCV (%)	Hb (g / dl)	TP (%)	TLC ($x10^3/mm^3$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
0	32.40 ± 2.56	10.76 ± 0.85	6.08 ± 0.26	1.85 ± 0.02	4.60 ± 0.75	95.40 ± 0.75	0.00 ± 0.43	0.00 ± 0.32	0.00 ± 0.30
1	36.00 ± 2.06	12.02 ± 0.67	5.92 ± 0.49	1.89 ± 0.01	6.40 ± 0.51	93.60 ± 0.51	0.00 ± 0.24	0.00 ± 0.25	0.00 ± 0.25
5	39.60 ± 1.63	13.22 ± 0.49	6.40 ± 0.45	1.90 ± 0.02	7.00 ± 1.05	92.80 ± 0.86*	0.00 ± 0.33	0.20 ± 0.43	0.00 ± 0.40
10	38.60 ± 2.60	12.82 ± 0.87	7.20 ± 0.42	1.92 ± 0.20*	7.80 ± 0.38*	91.80 ± 0.20**	0.00 ± 0.44	0.40 ± 0.25	0.00 ± 0.25

Superscript column wise on right hand side for particular treatment group are significantly ($P < 0.05$) different from the control group.
n = 5, TLC = total leucocyte count; PCV = packed cell volume; HB = hemoglobin count; TP = total protein.

CHAPTER FIVE

DISCUSSION

Results of the present study demonstrates that methanolic extract of *Mangifera indica* kernel revealed a good antibacterial activity. In this study, methanol extract showed antibacterial activity with size of inhibition zones against the test organism that ranges from 16 ± 2.41 to 24 ± 0.58 mm. The finding is in line with the findings of Khammuang and Sarnthima (2011), where they reported that different mango kernel extracts showed interesting antibacterial activity against both Gram positive and Gram negative bacteria as determine by disc diffusion method and the most sensitive strain inhibited by all extracts was *Pseudomonas aeruginosa*.

Acute toxicity test demonstrated that *Mangifera indica* kernel powder incoperated in feed at different doses used produced zero mortality in both the treatment and control groups. The LD_{50} is greater than 60 g/kg of feed with 99.9 % confidence in this study. The finding suggest that the mango kernel powder is safe to be incoperated into feed at 60 g/kg of feed and below.

Organic solvents are used to extract constituents of plants. The different polarities of plants secondary compounds make them more readily extracted by organic solvents of varying polarities. The use of organic solvent does not negatively affect the pharmacological activity of plant compounds. In this study, the methanol extract showed superior antibacterial activity when compared with kernel water solution, suggesting that organic solvents are clearly better solvents of antimicrobial agents present in *M. indica* kernel, which is in line with the findings of Thongson *et al.*, (2004) that methanolic extracts of

Thai spices provided the best antimicrobial effect because of some additional antimicrobial components that were extracted by organic polar solvents.

In this study, qualitative phytochemical analysis of the kernel revealed the presence of metabolites like triterpenes, condense and hydrolysable tannins, saponins and flavonoids. The presence and isolation of some phytoconstituents may lead to development of other useful drugs (Yakubu *et al.,* 2005). According to Engels *et al.* (2009), hydrolysable tannins isolated from mango kernels have shown an antibacterial activity as evidence from agar spot and critical dilution assays. Although growth of lactic acid bacteria was not inhibited, the proliferation of Gram-positive food spoilage bacteria was prevented and the growth of Gram-negative *E. coli* was reduced, but the bacterial growth was restored by addition of iron to the medium, which strongly supports the view that the inhibitory effects of hydrolyzable tannins are due to their iron-complexing properties. Saponins are characterized by their surface active properties and they dissolve in water to form foamy solutions and because of surface tension reducing activity some drugs containing saponins have been use as antibacterial drugs (Dean, 1999). Saponins have been shown to have antibacterial activity (Mandal *et al.,* 2005; Manjunatha, 2006). Similarly, many other flavonoids have been reported to exhibit antibacterial activity (Tereschuk *et al*., 1997), plants containing high concentrations of flavonoids are frequently reported to show antibacterial activity (Bosio *et al*., 2000). The antibacterial properties exhibited by *M. indica* kernel extract in this study, could therefore be attributed to the presence of steroidal saponins, flavonoids, tannins and other polyphenolic compounds. Polyphenols were earlier reported to have some antibacterial activities (Tomas-Barberan *et al.,* 1990), and the antibacterial effect of the different phytoconstituents of *M. indica* kernel may perhaps

present a synergistic action against the tested organism (*A. caviae*). Hammam (2007) reported that the antimicrobial activity of mango kernel extract is ascribed to seven major phenolic compounds assumed to be mainly ellagic acid, gallic acid, opigenin-oglucoside, mangiferin, methylgallate and pentagalloyl glucose. Several mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of cytoplasmic and plasma membranes, inhibition of extracellular microbial enzymes and metabolisms, and deprivation of the substrate required for microbial growth (Bell *et al*., 1965; Ikigai *et al*., 1993; Puupponen-Pimia *et al*., 2004).

The aqueous extract of *M. indica* kernel did show antibacterial activity against *A. caviae* at lower concentrations (166, 125, 100, 55.6 and 50 mg/ml) with smaller zones of inhibition that ranges from 8 ± 1.00 to 11 ± 1.00, with no measurable zones of inhibition at higher concentrations (250 and 500 mg/ml). This could be due to the inability of the aqueous extract to diffuse into the media at higher concentrations, due to the thickness of the solution of *M. indica* kernel at higher concentration of 250 and 500 mg/ml. However, the aqueous extract only inhibits growth of the test organism over short period of time, because the bacterial growth was restored eigth hours post incubation. This could be due to limited bacteriostatic effect of the aqueous extract on the *A. caviae*.

Serum obtained from fish treated with *M. indica* kernel has not shown antibacterial activity against the test organism. The finding in this study is not in aggreement with that reported by Thuy *et al.,* (2002) and Sahu *et al.,* (2007) where they reported that serum obtained from *Labeo rohita* treated with mango kernel had significant bactericidal effect against *A. hydrophila* when compared with the untreated (control group). The discrepancy could be

due to the use of test procedure (modified agar well diffusion method Perez *et al*., 1990) different from the one they used (procedure of Kajita *et al.,* 1990).

Studies have also shown that mango kernel contain various phenolic compounds that serve as good source of natural antioxidants (Soong and Barlow, 2004). Although serum obtained from fish treated with *M. indica* kernel and that from the untreated group did not inhibit the growth of *A. caviae*. It is possible that the *M. indica* kernel enhances the immune status of fish through antioxidant action. Studies by Sahu *et al.* (2007) did show that *M. indica* kernel enhances immune status by generating superoxide anion and its reactive derivatives (i.e. hydrogen peroxide and hydroxyl radicals). *M. indica* (mango) kernel when used as a feed additive increased the superoxide anion production in *Labeo rohita* fingerlings (Sahu *et al.,* 2007). Continuous feeding of raw mango kernel powder reduces the effects of stressors by increasing the level of glucose in circulation (Sahu *et al.,* 2007). Rohu fingerlings fed with mango kernel incoperated feed have higher serum albumin and globulin values than fingerlings fed with basal diet (Sahu *et al.,* 2007). Increases in serum protein, albumin and globulin levels are thought to be associated with a stronger innate response of fishes (Wiegertjes *et al.,*1996; Rao *et al.,* 2006; Sahu *et al.,* 2006).

The survivability of fish fed with mango kernel was higher when compared to fish fed with free mango kernel ration. The enhanced survival rate of fish fed with mango kernel ration could be due to enhance resistance of the fish to *A. caviae*. This could be due to the enhancement of the non-specific immune system of fish by mango kernel. Similar results were reported after feeding *Labeo rohita* fingerlings with *M. indica* kernel and challenged with *A. hydrophila* (Schaperclaus,1992; Sahu *et al*., 2007). Pachanawan *et al*. (2008) reported that the ethanol extract of *Psidium guajava* leaf fed to *Oreochromis niloticus*

caused enhanced survival rates when challenged with *A. hydrophila*. Similarly, Ardo *et al*. (2008) reported that feeding tilapia (*Oreochromis niloticus*) with two Chinese medicinal herbs (*Lonicera japonica* and *Ganoderma lucidum*) reduced mortality in fish experimentally infected with *A. hydrophila*.

There was significant difference ($p < 0.05$) between the neutrophil counts of fish treated with mango kernel at 10 g/kg of feed and untreated fish 40 days post-treatment. The result obtained is in corcodance with report by Sahu *et al.,* (2007) where significant difference occur between neutrophil counts of treated and untreated groups of *Labeo rohita (*rohu). This also supports the findings of Weeks and Warinner (1986) that increased in neutrophil could be an indicator of non-specific response. The WBC was significantly higher in fish treated with mango kernel at 10 g/kg of feed 40 days post-treatment. This result is also supported by another study (Choudhury *et al.,* 2005), who found that there was an increase in the WBC count when *L. rohita* juveniles were treated with immunostimulants like levamisole and ascorbic acid.

In a similar manner, there was no significant increase in the haemoglobin values of treated and untreated fish. The haemoglobin content of *Labeo rohita* fingerlings did not increase significantly when fed dietary mango kernel (Sahu *et al.,* 2007).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study has shown that phytochemical analysis of mango kernel revealed the presence of triterpenes, tannins, glycosides, saponins and flavonoids. The methanolic extract of mango kernel possesses antibacterial activity. However, the aqueous extract of mango kernel did produce reduced antibacterial effect against the test organism compared to that of methanol extract. The antibacterial activity of the *M. indica* kernel could be due to the presence of one or more of the secondary metabolites detected such as flavonoids, tannins, triterpenes and saponins which were earlier shown to have antibacterial effect if properly harnessed. *M. indica* kernel can be used as feed additive to protect against infection caused by motile aeromonads. The results demonstrated that the serum of fish treated with mango kernel at different concentrations produced no antibacterial effect against *A. caviae*. However, the total leukocyte counts were significantly higher in fish treated with mango kernel at 10 g/kg of feed 40 days post-treatment. Results obtained in this study also indicate that *M. indica* kernel protects *C. gariepinus* against *A. caviae* infection by enhancing the survivability of the treated fish.

In conclusion, the present study has revealed that long term dietary administration of mango kernel considerably increases WBC counts and survival of fingerlings of African catfish.

6.2 Recommendations

Detailed phytochemical studies (quantitative studies) to revealed active bioconstituents should also be undertaken. Furthermore, isolation and characterisation of active compound(s) contained in the plant is recommended. Further trials on other bacterial pathogens on target animal species should be undertaken as well. Mango kernel should be incooperated into fish feed as it has some beneficial effects.

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APPENDICES

APPENDIX I:

T- TEST

Group Statistics

	LEV	N	Mean	Std. Deviation	Std. Error Mean
CON	S	5	.0120	.01037	.00464
	T	4	260.2500	168.08802	84.04401

Independent Samples Test

		F	Sig.	T	df	Sig. (2-taile
CON	Equal variances assumed	8.193	.024	-3.525	7	
	Equal variances not assumed			-3.096	3.000	

APPENDIX II:

Parameter		
Table Analyzed	Row stats of HETROPHILS	1
One-way analysis of variance		
P value	0.1176	
P value summary	ns	
Are means signif. different? (P < 0.05)	No	
Number of groups	4	
F	2.288	
R squared	0.3002	
Bartlett's test for equal variances		
Bartlett's statistic (corrected)	4.706	
P value	0.1947	
P value summary	ns	
Do the variances differ signif. (P < 0.05)	No	

ANOVA Table	SS	df	MS
Treatment (between columns)	31.75	3	10.58
Residual (within columns)	74.00	16	4.625
Total	105.8	19	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	-0.4000	0.4159	No	ns	-4.291 to 3.491
A vs C	-0.6000	0.6239	No	ns	-4.491 to 3.291
A vs D	-3.200	3.327	No	ns	-7.091 to 0.6913
B vs C	-0.2000	0.2080	No	ns	-4.091 to 3.691
B vs D	-2.800	2.911	No	ns	-6.691 to 1.091

C vs D	-2.600	2.703	No	ns	-6.491 to 1.291

APPENDIX III:

Parameter					
Table Analyzed	Row stats of HETROPHILS2	2			
One-way analysis of variance					
P value	0.0371				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	3.592				
R squared	0.4025				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	4.142				
P value	0.2465				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	27.75	3	9.250		
Residual (within columns)	41.20	16	2.575		
Total	68.95	19			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column A vs Column B	-1.800	2.508	No	ns	-4.704 to 1.104

Column A vs Column C	-2.400	3.344	No	ns	-5.304 to 0.5036
Column A vs Column D	-3.200	4.459	Yes	*	-6.104 to -0.2964
Column B vs Column C	-0.6000	0.8361	No	ns	-3.504 to 2.304
Column B vs Column D	-1.400	1.951	No	ns	-4.304 to 1.504
Column C vs Column D	-0.8000	1.115	No	ns	-3.704 to 2.104

APPENDIX IV:

Parameter	
Table Analyzed	LYMPHOCYTES 1
One-way analysis of variance	
P value	0.0717
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	4
F	2.828
R squared	0.3465
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	4.860
P value	0.1824
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	34.15	3	11.38
Residual (within columns)	64.40	16	4.025
Total	98.55	19	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	0.0000	0.0000	No	ns	-3.630 to 3.630
A vs C	1.000	1.115	No	ns	-2.630 to 4.630
A vs D	3.200	3.567	No	ns	-0.4301 to 6.830
B vs C	1.000	1.115	No	ns	-2.630 to 4.630
B vs D	3.200	3.567	No	ns	-0.4301 to 6.830
C vs D	2.200	2.452	No	ns	-1.430 to 5.830

APPENDIX V:

Parameter	
Table Analyzed	Row stats of LYMPHOCYTES 2
One-way analysis of variance	
P value	0.0070
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	5.800
R squared	0.5210
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	6.454
P value	0.0915
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS

Treatment (between columns)	34.80	3	11.60	
Residual (within columns)	32.00	16	2.000	
Total	66.80	19		

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	1.800	2.846	No	ns	-0.7589 to 4.359
A vs C	2.600	4.111	Yes	*	0.04108 to 5.159
A vs D	3.600	5.692	Yes	**	1.041 to 6.159
B vs C	0.8000	1.265	No	ns	-1.759 to 3.359
B vs D	1.800	2.846	No	ns	-0.7589 to 4.359
C vs D	1.000	1.581	No	ns	-1.559 to 3.559

APPENDIX VI:

Parameter	Row stats of TLC
Table Analyzed	1
One-way analysis of variance	
P value	0.0932
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	4
F	2.538
R squared	0.3224
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	2.824
P value	0.4196
P value summary	ns

Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	0.01142	3	0.003807
Residual (within columns)	0.0240	16	0.0015
Total	0.03542	19	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	-0.008000	0.4619	No	ns	-0.07808 to 0.06208
A vs C	-0.008000	0.4619	No	ns	-0.07808 to 0.06208
A vs D	-0.06000	3.464	No	ns	-0.1301 to 0.01008
B vs C	0.0000	0.0000	No	ns	-0.07008 to 0.07008
B vs D	-0.05200	3.002	No	ns	-0.1221 to 0.01808
C vs D	-0.05200	3.002	No	ns	-0.1221 to 0.01808

APPENDIX VII:

Parameter	Row stats of TLC
Table Analyzed	2
One-way analysis of variance	
P value	0.0192
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	4.412

R squared	0.4527					
Bartlett's test for equal variances						
Bartlett's statistic (corrected)	2.154					
P value	0.5410					
P value summary	ns					
Do the variances differ signif. (P < 0.05)	No					
ANOVA Table	SS	df	MS			
Treatment (between columns)	0.01350	3	0.004500			
Residual (within columns)	0.01632	16	0.00102			
Total	0.02982	19				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff	
A vs B	-0.03600	2.521	No	ns	-0.09379 to 0.02179	
A vs C	-0.04800	3.361	No	ns	-0.1058 to 0.009788	
A vs D	-0.07200	5.041	Yes	*	-0.1298 to -0.01421	
B vs C	-0.01200	0.8402	No	ns	-0.06979 to 0.04579	
B vs D	-0.03600	2.521	No	ns	-0.09379 to 0.02179	
C vs D	-0.02400	1.680	No	ns	-0.08179 to 0.03379	

APPENDIX VIII:

Parameter	Row stats of PCV				
Table Analyzed	1				
One-way analysis of variance					
P value	0.1908				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	4				
F	1.783				
R squared	0.2506				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	2.959				
P value	0.3979				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	176.6	3	58.85		
Residual (within columns)	528.0	16	33.00		
Total	704.6	19			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	-4.400	1.713	No	ns	-14.79 to 5.994
A vs C	-6.200	2.413	No	ns	-16.59 to 4.194
A vs D	-8.000	3.114	No	ns	-18.39 to 2.394
B vs C	-1.800	0.7006	No	ns	-12.19 to 8.594

B vs D	-3.600	1.401	No	ns	-13.99 to 6.794
C vs D	-1.800	0.7006	No	ns	-12.19 to 8.594

APPENDIX IX:

Parameter	Row stats of PCV
Table Analyzed	2
One-way analysis of variance	
P value	0.1463
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	4
F	2.058
R squared	0.2784
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	0.9868
P value	0.8045
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	154.9	3	51.65
Residual (within columns)	401.6	16	25.10
Total	556.5	19	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff

A vs B	-3.600	1.607	No	ns	-12.67 to 5.465
A vs C	-7.200	3.214	No	ns	-16.27 to 1.865
A vs D	-6.200	2.767	No	ns	-15.27 to 2.865
B vs C	-3.600	1.607	No	ns	-12.67 to 5.465
B vs D	-2.600	1.160	No	ns	-11.67 to 6.465
C vs D	1.000	0.4463	No	ns	-8.065 to 10.07

APPENDIX X:

Parameter			
Table Analyzed	Row stats of Hb 1		
One-way analysis of variance			
P value	0.1887		
P value summary	ns		
Are means signif. different? (P < 0.05)	No		
Number of groups	4		
F	1.795		
R squared	0.2518		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	2.965		
P value	0.3970		
P value summary	ns		
Do the variances differ signif. (P < 0.05)	No		
ANOVA Table	SS	df	MS
Treatment (between columns)	19.50	3	6.499
Residual (within columns)	57.93	16	3.621

Total	77.43	19			

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	-1.480	1.739	No	ns	-4.923 to 1.963
A vs C	-2.060	2.421	No	ns	-5.503 to 1.383
A vs D	-2.660	3.126	No	ns	-6.103 to 0.7830
B vs C	-0.5800	0.6816	No	ns	-4.023 to 2.863
B vs D	-1.180	1.387	No	ns	-4.623 to 2.263
C vs D	-0.6000	0.7051	No	ns	-4.043 to 2.843

APPENDIX XI:

Parameter	
Table Analyzed	Row stats of Hb 2
One-way analysis of variance	
P value	0.1322
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	4
F	2.164
R squared	0.2886
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	1.436
P value	0.6970
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	17.65	3	5.885
Residual (within columns)	43.52	16	2.720
Total	61.17	19	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
A vs B	-1.260	1.708	No	ns	-4.244 to 1.724
A vs C	-2.460	3.335	No	ns	-5.444 to 0.5240
A vs D	-2.060	2.793	No	ns	-5.044 to 0.9240
B vs C	-1.200	1.627	No	ns	-4.184 to 1.784
B vs D	-0.8000	1.085	No	ns	-3.784 to 2.184
C vs D	0.4000	0.5424	No	ns	-2.584 to 3.384