IN VITRO AND *IN VIVO* ANTICOCCIDIAL ACTIVITIES OF EXTRACTS OF *KHAYA SENEGALENSIS* (DESR. A. JUSS.) AGAINST *EIMERIA TENELLA* IN BROILER CHICKENS

BY

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DECLARATION

I hereby declare that the work in this thesis titled "***In vitro* and *in vivo* anticoccidial activities of extracts of *Khaya senegalensis* (Desr. A. Juss.) Against *Eimeria tenella* in Broiler Chickens"** was carried out by me in the Department of Veterinary Parasitology and Entomology under the supervision of Profs. I.A. Lawal, B.D.J. George and M.S. Abubakar. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented elsewhere for a degree or diploma at any other institution.

Name of student	**Signature**	**Date**

CERTIFICATION

This thesis titled ***"IN VITRO* AND *IN VIVO* ANTICOCCIDIAL ACTIVITIES OF EXTRACTS OF *KHAYA SENEGALENSIS* (DESR. A. JUSS.) AGAINST *EIMERIA TENELLA* IN BROILER CHICKENS"** by **Martha Onyiohinoyi Otu** meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) of Veterinary Parasitology of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to God Almighty for seeing me through.

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ABSTRACT

A study to determine the *in vitro* and *in vivo* anticoccidial activities of *Khaya senegalensis* leaf extracts on *Eimeria tenella* infection in broilers was conducted. A preliminary investigation was carried out using three plants namely; *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica* to select the one with the most anticoccidial activity. The leaves were extracted by maceration method for 72 hours using 70% methanol and the partial purification of the dried crude methanol extracts done. The separated portions of the extracts were then evaluated by Thin Layer Chromatography (TLC). The chromatography result showed that *Khaya senegalensis* has phenols and flavonoids. The n-butanol fractions were tested for *in vitro* anticoccidial activities on the sporulation time of the *Eimeria tenella* oocysts. *Khaya senegalensis* n-butanol fraction had the most anticoccidial activity in which there was total sporulation inhibition (100%) while *Guiera senegalensis* had the least anti coccidial activity with 49% sporulation inhibition. The maximum tolerated dose (MTD) was determined using graded doses of the n-butanol extracts of *Khaya senegalensis* at 11 mg/kg, 33 mg/kg, 99 mg/kg, 297 mg/kg and 891 mg/kg administered orally to 4 weeks old birds at the same period which were divided into groups A- E respectively while group F birds were given 0.2 ml/bird of distilled water as control. The high survival rate of 100% in the five treated groups of the maximum tolerated dose experiment indicated the high safety margin of *Khaya senegalensis* even at a dose of 891mg/kg. The progressive weight increase in both the treated (11mg/kg) and un treated group shows the ability of the extract to improve weight gain. The histopathological lesions had some degree of nephro-toxicity seen. A chronic toxicity study was also conducted using the n-butanol portion of the extracts of *Khaya senegalensis*. Two groups of 7 birds each (1 and 2) of 4 weeks of age broilers were used with group 1 chickens orally dosed with 500 mg/kg of the n-butanol fraction of the extract daily for

two weeks. Group 2 served as the control group and each bird was given distilled water at 0.2ml. The gross lesions showed that 500 mg/kg given over a two weeks period had effects on the internal organs of the birds as revealed by the hypertrophied, congested kidneys. Evaluation of the modulatory effect of the n-butanol fraction of the crude methanol extracts of *Khaya senegalensis* against *E tenella* in broilers was carried out using one hundred and twenty, 4 week-old birds which were obtained from the National Animal Production and Research Institute (NAPRI), Shika, Zaria. The higher mean oocyts count in the infected untreated group (48%) than the treated group two weeks post infection indicated that the extract had some inhibitory effects on oocyst count. Among the extract treated birds, the high oocyst count by the 11 mg/kg group (17%) suggests the dose was not adequate to prevent oocyst production while the 99 mg/kg had lower oocyst counts (12%) which was comparable to the group treated with normal dose of Amprolium (10%) and thus was the effective dose 2 weeks post treatment. Also the phenols at a dose of 99mg/kg used to treat the infected birds three weeks post treatment, reduced oocysts count by 2%. The presence of schizonts and merozoites in caecal scrappings showed that reproductive activities of the parasites were not completely halted even after treatment. It also shows that the endogenous stages were not susceptible to the extracts at all doses although the population in the treated infected groups were lower than those seen in the infected un treated group. The survival percentage was higher in the treated groups compared to the infected un treated group (55%). Among the treated groups, the survival rate was numerically higher in the 33 mg/kg (85%) treated group while the least was in the group treated with 99 mg/kg (70%). The infected groups had slightly lower Packed Cell volume (PCV) even after treatment with the extracts than the uninfected or Amprolium treated group. In this experiment the lowest PCV was 24.66% at the third week post infection in the 33mg/kg treated group. The histopathological

lesions observed included very severe lesions including severe villous atrophy and fusion of the villi. In conclusion, the *in vitro* and *in vivo* modesl were found to be useful tools for rapid screening of anticoccidial activity of the plant preparations against *Eimeria tenella* parasites. It is recommended that recommended that extract of *Khaya senegalensis* can be given at a dose of 11 mg/kg as a natural growth promoter. Also anticoccidial *in vitro* activity of *K senegalensis* as it relates to *in vivo* anticoccidial activity is an interesting area for more research. Detailed studies are needed to isolate, characterize and evaluate the active components and the mechanism of action of the identified active principles in *Khaya senegalensis* leaves as well as other parts of the plant.

Chapter One

1.0 Introduction

Coccidiosis is one of the most detrimental and lethal management diseases of poultry (Gyorke *et al*., 2013; Khan *et al*., 2008). Chickens are susceptible to at least nine (9) species of Coccidia (but only five are pathogenic) (Jordan, 1990). The most common and pathogenic species are *Eimeria tenella* which causes caecal coccidiosis and *Eimeria necatrix* (Gyorke *et al*., 2013), while *Eimeria acervulina* and *Eimeria maxima* cause chronic intestinal coccidiosis (Chandrakesan *et al*., 2009). The disease may be acute or chronic (Takagi *et al*., 2006) and is associated with haemorrhagic diarrhoea, emaciation, growth retardation and sometimes death (Takagi *et al*., 2006). Birds of any age may be affected but problems are not common in chicks under two weeks of age (Wright, 2005).

Poultry keeping is the dorminant form of livestock production in developing countries (Numan *et al*.,2005). In Nigeria, the rising cost of poultry feeds, problems of drug residues and incubial resistance to diseases have become major problems militating against the poultry industry (Ogbe *et al*., 2008). Porter (1998) estimated that feed for poultry comprise roughly 66 % of the total production costs for layer, broiler chickens or turkeys. In addition, severe enteric damage by bacterial infection will cause overt illness and high mortality in a poultry flock (Elmulsharaf *et al*., 2006). Coccidiosis has remained the most important poultry disease in Nigeria (Obasi *et al.*, 2006). Lawal *et al.* (2008) showed that the infection can occur in both local chickens and exotic birds with the former serving mainly as reservoir hosts. Management systems (deep litter, cage and free range) have also been shown to be of no barriers to the occurrence of avian coccidiosis in Zaria (Lawal *et al.*, 2001). The disease causes considerable economic losses in both the layer and broiler industries (Al-Quraishy *et al*., 2009; Chandrakesan *et al*., 2009; Yin *et al*., 2013).

Anticoccidial drugs have been in use for more than 50 years and presently almost all commercial broilers are reared with the agents in their feeds (Chapman, 2001; Chandrakesan *et al*., 2009). The development of drug- resistant field strains and the necessity for withdrawal period prior to slaughter prompted the exploitation of alternative methods for controlling coccidiosis (Zulpo *et al*., 2007). Consequently, due to the ban on antimicrobial growth promoters (AGP), the use of plant extracts has gained attention in animal feed strategies and formulations (Tipu *et al*., 2006).

There is an increasing use of medicinal plants and plant products as alternatives to orthodox medicine especially in developing nations (Mikail and Ajabonna, 2007). Plants native to Nigeria have been experimented and shown to have some anti coccidial activities (Anosa and Okoro, 2011) with the bark of *Khaya senegalensis* used to treat coccidiosis, helminthosis and diarrhoea in poultry (Gefu *et al*., 2000). Similarly, *Tamarindus indica* works as a purgative, diaphorectic and an anthelminthic and is also said to have anti trypanosomal activity (McCorkle *et al*., 1996; Gefu *et al*., 2000). *Gueira senegalensis* is widely used in traditional medicine in West Africa (Males *et al.,* 1998) and is known to have antimicrobial effects (Sanogor *et al.,* 1997). *Khaya senegalensis*, *Gueira senegalensis* and *Tamarindus indica* are widely used as medicinal plants in West Africa (Males *et al.,* 1998) and are also known to have antimicrobial effects (Sanogor *et al.,* 1997).

This study is being carried out to determine the possible effects of the leaf extracts of the plants named above on the causative agents of avian coccidiosis and to compare them with the standard conventional treatment.

1.1 Statement of Research Problem

Host specific coccidiosis is caused by *Eimeria* species which belong to the Phylum Apicomplexa, Family Eimeridae and genus *Eimeria.* It is the major parasitic disease of poultry causing substantial economic losses due to malabsorption, bad feed conversion rate, reduced weight gain and mortality (Fornace *et al*., 2013). Although some clinical eimerial infections may be unimportant (Gerhold Jr, 2014), they can cause considerable depression in feed efficiency and egg production, which accounts for 70% of the total coccidiosis disease control cost. Coccidiosis is a disease of poor management and mortalities due to the disease are ranked second after viral diseases of poultry (Hadipour *et al*., 2013). The disease is responsible for 6-10% of all broiler mortalities and incidence of the disease in commercial poultry ranges from 5-70% (Usman *et al.,* 2011). The poultry industry is facing problems which include drug resistance, lack of new anticoccidial products and consumer demanding decrease in the use of drugs in animal feeds (Hafez, 2008). The extensive use of prophylactic anticoccidial drugs has led to the development of drug resistant strains of *Eimeria* against all products introduced (Chapman, 1997). Live vaccines can restore drug sensitivity and proved to be efficient in controlling the disease but they are expensive and have adverse effects on early chick growth (Williams, 2002). Also the control of coccidiosis in replacement birds namely broiler breeders and egg producing stock is a continuing problem since most anticoccidials cannot be given to birds in egg production , yet most adult birds live in an infected environment (Usman *et al.,* 2011). This suggests that coccidiosis is likely to have a greater impact on the profitability of broiler meat and egg productions in future (Sunhwa *et al.,* 2014). There is therefore an urgent need to seek alternative strategies to control coccidiosis.

1.2 Justification

Viral and protozoan diseases are the most outstanding infectious diseases militating against productive poultry industries in Nigeria (Abdu *et al*., 2007). Prominent among the protozoan diseases is coccidiosis. Coccidiosis is a protozoan disease commonly occurring under intensive management system (Biu *et al.*, 2006) where pathogenic population of the causative agent *Eimeria* specie (Obasi *et al*.,2006) easily builds up causing significant economic losses through reduction in weight, feed consumption and mortality (Gyorke *et al*.,2013). It currently represents one of the greatest challenges to the broiler industry especially in the production of broiler chickens (Bliss, 2007). The economic losses are significant being estimated at more than 3 billion US dollars annually worldwide (Gyorke *et al*., 2013). In the United States, well over 600 million dollars are lost annually due to coccidiosis (Bliss, 2007). Although coccidiosis is known to be endemic in Nigeria, there is a dearth of accurate data on the economic impact (Obasi *et al.*, 2006). There are records of losses due to the disease at clinical and sub clinical levels and cost of control measures are appreciable and reprehensible (Obasi *et al.*, 2006; Mikail and Ajabonna, 2007).

In recent times, there has been increasing global interest in ethno- medical and ethno-veterinary practices especially as it relates to the use of medicinal plants in treating various ailments. In the developed world, this move is in response to the production of animals free from industrial chemical inputs and the need to discover new therapeutic substances of natural origin with possibly low toxicity to man and animals. In Africa this is borne out of economics and affordability (Alawa *et a*l., 2003; Tipu *et al*., 2006).

Khaya senegalensis is a well known medicinal plant (Sale *et al*., 2008; Ndjonka, *et al*., 2011; Kolawole, *et al*., 2012; Ibrahim *et al*., 2013) and the active compounds of *Khaya senegalensis* have been described by Yaun *et al*. (2013) and Nakatani *et al*. (2002). Proximate analysis of *K. senegalensis* leaves revealed that they are rich in dry matter, organic matter, minerals, gross energy and crude protein (Abdel-Wareth *et al.*, 2014). In Nigeria, the bark of *Khaya senegalensis* is used to treat coccidiosis, helminthosis, amoebic dysentery and diarrhoea in poultry (Gefu *et al*., 2000). It has also been reported to have some therapeutic effects against *Trypanosoma* species (Otu *et al.*, 2009). Anti oxidants and flavonoids are possible candidates in natural products which occur naturally (Taheri *et al*., 2005) with many medicinal plants been good sources (Emami, 2007) and are stored in different parts of some plants (Bhakuni *et al*., 2001; Brisibe *et al*., 2009). The identification of phenolic compounds in *Khaya senegalensis* which are also antioxidants has been indicated to have anticoccidial activities (Naidoo *et al*., 2008; Meskerem and Boonkaewwan, 2013). Oxidants can be produced at elevated rates under patho-physiological conditions (Meskerem and Boonkaewwan, 2013). Phenolic compounds could be a major determinant of antioxidant potentials of foods and are therefore a natural source of antioxidants (Aberoumand and Deokule, 2008). However, the effects of leaf extracts of the plants on Coccidia, another important protozoan parasite is not known. The present study is therefore conceived to bridge this gap of knowledge and probably contribute towards finding solutions to effective treatment and control of avian coccidiosis in Nigeria.

1.3 Aim of the study:

The aim of the study is to determine the chemical constituents and anticoccidial activities of extracts of *Khaya senegalensis* leaves using experimental *Eimeria* infection in broilers.

1.4 Objectives of the study:

The objectives of the study are to

i: Fractionate the methanol extracts of *Khaya senegalensis*, *Guiera senegalensis* and *Tamarindus indica*

ii: Evaluate the *in vitro* anticoccidial effect of the extracts of *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica.*

iii: Evaluate the *in vivo* anticoccidial effect of the extracts.

1.5 Research Question

Do aqueous and n-butanol fraction of the crude methanol extracts of *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica* have anticoccidial effects against *Eimeria tenella* infection in broilers *in vivo* and *in vitro*?

Chapter Two

2.0 Literature Review

2.1 Avian cocciodiosis

Avian coccidiosis is one of the most common and important diseases in poultry production caused by protozoan parasites of the genus *Eimeria* (Perez – Carbajal *et al*., 2010). Host specific coccidiosis is the major parasitic disease of poultry causing substantial economic losses due to malabsorption, bad feed conversion rate, reduced weight gain and mortality (Fornace *et al*., 2013). In poultry, most species of *Eimeria* infect various sites in the intestine (Gerhold Jr, 2014). Seven species of *Eimeria* have been recognized to specifically parasitize the domestic chickens (*Gallus gallus*) with differing levels of pathogenicity and specific areas of localization within the digestive tract (Fornace *et al*., 2013). The disease is characterized by enteritis and diarrhoea which can become bloody with certain *Eimeria* species (Dakpogan and Salifou, 2013). The replication of *Eimeria* sporozoites in the epithelial cells of the intestinal tract causing tissue damage with resulting interruption of digestive processes or nutrient absorption, dehydration and blood loss (Dakpogan and Salifou, 2013). The macroscopic lesions in the digestive tract predispose the infected birds to many gastrointestinal bacterial poultry diseases such as clostridiosis, salmonellosis and collibacillosis (Bostrironnois and Zadjian, 2011). Certain immunosuppressive viral diseases such as infections bursal disease, Marek's disease and Chick anaemia infectious viral disease also exacerbate coccidiosis (Lanckriert *et al*., 2010). Coccidiosis is a disease of poor management (Dakpogan and Salifou, 2013); mortalities due to the disease are ranked second after viral diseases of poultry (Hadipour *et al*., 2013).

2.1.1 The Causative Agents of Avian Coccidiosis

Coccidiosis is caused by *Eimeria* species which belong to the Phylum Apicomplexa, Family Eimeridae and genus *Eimeria* (Amin *et al*., 2014). They are intracellular protozoan parasites (Lillehoj and Trout, 1996). Nearly 1800 *Eimeria* species have been reported affecting the intestinal mucosa of different animals and birds (Al Natoura *et al.,* 2002; Hamidinejat *et al*., 2010; Amin *et al*., 2014). Among these, nine *Eimeria* species have been reported to affect the domestic fowls (Bachaya *et al*, 2012; Perez-Carbajal *et al*., 2010; Nematollahi *et al*, 2009). These include the highly pathogenic species identified as *Eimeria tenella, E maxima, E brunette* and *E necatrix* (Amin *et al*., 2014). *E acervulina, E mivati* and *E mitis* are reported as less pathogenic and *E hagani* and *E praecox* reported as mild (Amin *et al*., 2014). Among the seven species of *Eimeria* commonly detected in infected chickens, *E tenella* is one of the most virulent and the only one for which the genome has been sequenced (Rieux *et al*., 2012).

Eimeria tenella is one of the most ubiquitous (Patra *et al*., 2010) and most pathogenic (Abbas *et al*., 2008). It is a significant cause of intestinal disease in chickens worldwide (Cacho *et al*., 2014). It is an intracellular obligate protozoan parasite (Abbas *et al*., 2008) having a complex life cycle of seven days (Cacho *et al*., 2014) during which it under-goes intracellular development and proliferates through characteristic intracellular stages confined to the caecal epithelium (Cacho *et al*., 2014). It causes caecal coccidiosis (Amin *et al*., 2014).

Coccidia are almost universally present in poultry raising operations, but clinical disease occurs only after ingestion of relatively large numbers of sporulated oocysts by susceptible birds (Gerhold Jr, 2014). Both clinically infected and recovered birds shed oocysts in their droppings

which contaminate feed, dust, water, litter and soil (Gerhold Jr, 2014). The infective form of the parasite is the oocyst which sporulates two days after following excretion through the faeces in the natural environment and can be ingested by a susceptible host (Dakpogan and Salifou, 2013).

Eimeria tenella inhabits the caeca (rarely adjacent intestinal tissues) causing hemorrhage and inflammation (Meskerem and Boonkaewwan, 2013). It is known as caecal or bloody coccidiosis and can be recognized by the accumulation of blood in the caeca and by bloody droppings (Meskerem and Boonkaewwan, 2013).

2.2 Morphology

The protozoan parasites may be found in poultry operations and exists as a highly resistant oocyst in litter. The oocysts vary in size with *E maxima* being the largest (about 20 × 30µ) and *E mitis*, the smallest (about 14×16 µ). Reid and Long (1979) established that oocysts of *Eimeria* species cannot be distinguished simply using their morphology. The oocysts are categorized according to size with minute variations in length and width. These categories are small, medium and large (Price and Barta, 2010).

i) The small category includes:

ii) *E. acervulina*

iii) *E. mitis*

iv) *E. mivati*

Medium size category:

i) E. necatrix ii) E. praecox

iii) E. tenella iv) E. brunetti

C. Large size: *E. maxima*

2.3 Life cycle

The life cycle of *Eimeria* comprises intracellular, extracellular, asexual and sexual stages (Min *et al*., 2004; Kitandu and Juranova, 2006). It does not require intermediate host (Chauhan and Roy, 2007) and also has a self limiting life cycle characterized by high tissue and host specificity (Hafez, 2008). The life cycle can also be divided into two distinct phases:

Internal phase (schizogony and gametogony) in which the parasite multiplies in different parts of the intestinal tract and the oocysts are excreted in the faeces (Hafez, 2008; Price and Barta, 2010).

The external phase (sporogony) during which the oocysts undergoes a final process called sporulation before they are infective. Sporulation requires warmth (25-30^{0}C), moisture and oxygen (Hafez, 2008). Unsporulated oocysts are released to the environment via the faeces of poultry (Prince and Barta, 2010). The unsporulated oocyst is non infective; however, after approximately 48 hours at optimum temperature (30^{0}C) and oxygen tension, the oocysts sporulate (Price and Barta, 2005). The sporulated oocyst is tetrasporic and dizoic with four sporocysts that contain two infective sporozoites within them (Price and Barta, 2010). The infective oocysts can remain viable in the litter for an extended period (months to years under ideal condition) until transmitted faecal-orally to a definitive host (Price and Barta, 2010).

After uptake of oocyts by the bird, sporozoites are released, which enter the gut epithelial cells. The invasion may occur 1-6 hours after the oocysts are ingested and form first generation

schizonts two days after infection (Klinkenberg and Heesterbeek, 2005). The grinding action of the gizzard releases the sporocysts from the oocysts while enzymatic action of trypsin, bile and carbon dioxide in the upper intestinal tract releases the sporozoites from the sporocysts (Allen and Fetterer, 2002). The sporozoites infect intestinal host epithelial cells in the gut linnings in various regions depending on the species of *Eimeria* (Price and Barta, 2010). The invasion of host cells by *Eimeria* specie is a complex multistep process that begins with the apical attachment of the parasite to the host cell (Jiang *et al*., 2012). This is followed by rapid internalization to form an intracellular, parasitophorous vacuole (PV) in which the newly invaded parasites becomes enclosed enabling its survival within the host (Tabares *et al*., 2004). The PV serves to protect the parasite against lysis by the host cell and constitutes a barrier between host cell metabolites (Entzeroth *et al*., 1998). During the invasion process, specialized secretory organelles known as micronemes, rhoptries and dense granules in the parasite deliver cargo proteins in a coordinate fashion (Jiang *et al*., 2012). The secreted proteins are thought to have a central role in the invasion and establishment of infection (Daszak, 1999; Jiang *et al*., 2012). Apical membrane antigen I (AMA1) which is secreted by micronemes is involved in the formation of the moving junction complex which is a circumferential zone that moves backward and eventually pinches the PV from the host cell membrane (Jiang *et al*., 2012). Recent developments in genomic and proteomic research have led to further insights into AMA1 from *Eimeria* specie. Expressed Sequenced Tags (ESTs) of *Eimeria tenella* were analyzed and some ESTs sequences showed homology with AMA1 (Lal *et al*., 2009). The AMA1 protein was also detected in sporozoites by proteomic comparison of four *E tenella* life cycle stages (Jiang *et al*., 2012). Within the host epithelial cells, the sporozoites form a trophozoite (round bodies having a nucleus) that undergoes merogony (asexual divisions) to form an immature meront; schizont

(Price and Barta, 2010). Sporozoites of some species (*E brunette* and *E praecox*) develop within cells at site of penetration. Sporozoites of other species (*E acervulina, E maxima, E necatrix* and *E tenella*) are transported to other sites (Allen and Fetterer, 2002). The nuclei of the first generation schizonts develop into sickle shaped bodies called merozoites. The merozoites are placed closely packed and parallel to each other looking somewhat like segments of orange (Chauhan and Roy, 2007). The merozoites lyse out of the original infected intestinal epithelial cells to infect new epithelial cells completing a second cycle of merogony (Innes and Vermeulen, 2006). These second generation of schizonts are formed the fourth day after infection which may be followed by the formation of third or fourth generation schizonts depending on the *Eimeria* species (Klinkenberg and Heesterbeek, 2005). At the conclusion of the last cycle of merogony, the resulting merozoites enter new host intestinal epithelia cells and initiate gametogony, the sexual phase of the life cycle (Price and Barta, 2010). In gametogony, the merozoites either become microgamonts that form microgametes (male gametes) or they become macrogamonts that mature to female macrogametes (Price and Barta, 2010). Merozoites that form a microgamont undergo multiple divisions that ultimately result in the production of many biflagellate microgametes, whereas merozoites that form a macrogamete mature to a single large macrogamete (Price and Barta, 2010). Microgametes will invade macrogamete infected host epithelial cells (Jeurissen *et al*. 1996). The resulting zygote (fertilized macrogamete) quickly forms an oocyst wall and turns into unsporulated oocyst which is shed by the chicken in the faeces on the eighth day after infection (Klinkenberg and Heesterbeek, 2005). Prepatent periods may generally range from 4 to 5 days post infection. Maximum output ranges from 6 to 9 days post infection (Allen and Fetterer, 2002; Price and Barta 2010). *Eimeria tenella* initially invades the epithelial cells linning the lumen of the Crypts of Lieberkhun (Cacho *et al*., 2014). The

ensuring life cycle of the parasites (Plate 2.1) involves detachment of the parasitized cells from the epithelial layer and their migration to the underlying connective tissue of the lamina propria (LP) where maturation to schizonts occur (Cacho *et al*., 2014). Early development of schizonts is accompanied by considerable hypertrophy and the modification of the host cell which survives in the LP as long as the schizont matures (Cacho *et al*., 2014). Schizont maturation takes place as the host cells harbouring the parasites migrate through the LP deep into the muscularis mucosa, the boundary between the LP and the submucosa (Cacho *et al*., 2014).

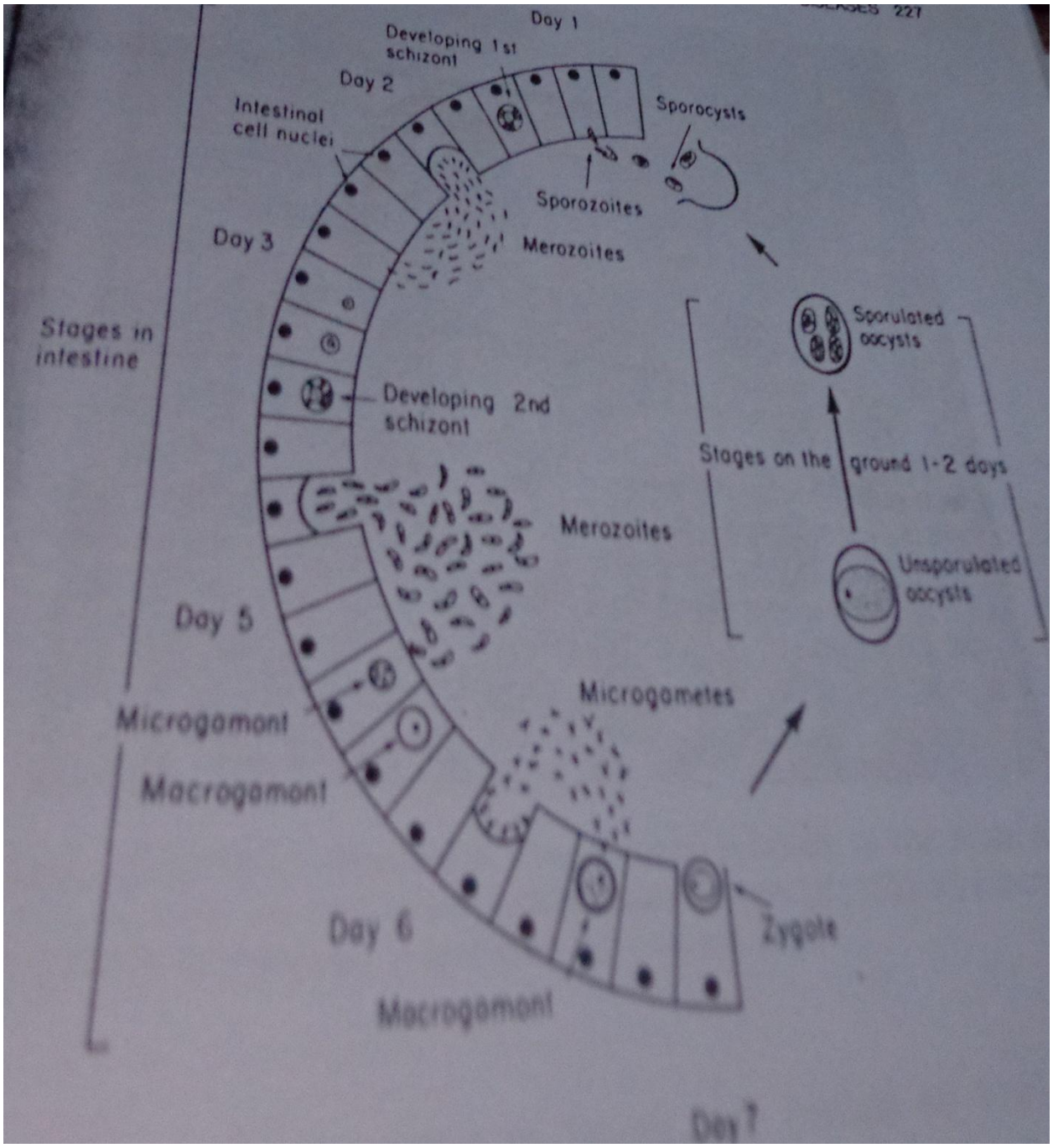


Plate 2.1: Life cycle of *Eimeria tenella* (Courtsey Jordan, 1990)

2.4 Coccidiosis in other species of birds

2.4.1 Turkey

Five species have been described which may cause lesion in turkeys. These species are listed in Table 2.1.

Table 2.1: Species of *Eimeria* in Turkeys

Parasite	Site of Lesion	Type of Lesion	Pathogenicity
Eimeria adenoides	Caecum	Possibly petechial,haemorrhage,mucoid exudates,caecal cores	High
E meleagrimitis	Anterior and mid intestine	Haemorrhagic lesion,dilation of jejunum,casts	High
E gallopovonis	Rectum	Ulceration, yellow exudates	High
E meleagridis	Caecum	Creamy exudates,caseous core	Low
E dispersa	Anterior and mid intestine	Watery or mucoid exudates,yellowish faeces	Low

They are thought to be widely distributed. They cause disease in young poults with older turkeys very resistant (Jordan, 1990). Dysentery is rarely seen, while watery diarrhoea is a more typical occurrence. *E adenoides* is the most pathogenic of turkey coccidia and causes high mortality in very young poults. In all these species, gamonts and oocysts are the stages most likely to be associated with lesions (Jordan, 1990).

2.4.2 Geese

Intestinal coccidiosis can be a severe problem in goslings (Jordan, 1990) and *E anseris* is the most pathogenic species with *E nocens* also a frequent concurrent infection (Jordan, 1990). Sign

of *E anseris* infection is haemorrhagic enteritis in the small intestine with diarrhoea (Jordan, 1990). Also highly pathogenic in goslings is *Eimeria truncata* which causes renal coccidiosis in goslings (Jordan, 1990). They parasitize the epithelial cells in the kidney tubules. Severe infection in 3-12 weeks old birds causes depression, emaciation, diarrhoea and mortality (Jordan, 1990).

2.4.3 Ducks

The most pathogenic coccidia infection is said to be caused by *Tyzzeria perniciosa* (Jordan, 1990). This causes severe diseases in ducklings less than 7 weeks of age with haemorrhagic enteritis of the anterior small intestine, dysentery and a high mortality (Jordan, 1990). The sporulated oocysts contain 8 sporozoites free within the oocysts.

2.4.4 Game Birds

In pheseants, the major species are *E colchici*, *E duodenalis* and *E phasiani*, with *E colchici* being the most pathogenic. They affect the small intestine and caeca. Acute disease with mortality may be associated with caecal coccidiosis caused by *E legionensis* in red-legged partridges and *E tsunodai* in Japanese quail. In young guinea fowls, coccidiosis due *E numidae* and *E grenieri* may be a problem (Jordan, 1990).

The morphological features and pathogenicity of Eimeria species found in birds is presented in table 2.2.

Table 2.2: Morphology and Pathogenicity of Eimeria species of birds

Species of *Eimeria*	Intestinal part/Location	Post mortem lesions	Characteristics of oocysts Size(um) mean length	Mean width	Shape index	Shape
E. tenella	Caeca	Haemorrhages and clotted blood in caecal pouches	17.20-24 .08	13.76 -20.64	1.21	Ovoid
E. necatrix	Middle small intestine	Ballooning of intestine mucoid blood filled exudates	17.20-24.08	13.76 -20.64	1.23	Oblong/ ovoid
E. acervulina	Anterior small intestine	Transverse whitish band on duodenal loop	13.76-20.64	10.32 -17.20	1.27	Ovoid
E. brunetti	Posterior small intestine	Mucoid bloody enteritis	20.64-27.52	17.52 24.08	1.22	Ovoid
E. maxima	Middle small intestine	Thickened intestinal wall petechiae	24.08-37.84	17.20-27.52	1.32	Ovoid

Courtsey by Adhikari *et al*., 2008

2.5 Transmission

The *Eimeria* species is ubiquitous, and survives in infected birds and environment for a long time (Shojaei, 2014). The oocyst can remain viable in poultry litter for many months (Hafez, 2008). However, they can be damaged by bacteria and other organisms and their viability can begin to diminish after three weeks (Allen and Fetterer, 2002). The oocysts have cyst walls that are highly refractory to environmental extremes and disinfectants and can be transported mechanically by

animals, insects, dust and contaminated feed, water and other formites (Shojaei, 2014). Direct oral transmission is the natural route of infection (Hafez, 2008). Bad management (such as wet litter, contaminated drinkers and feeders, bad ventilation and high stocking density) can exacerbate the clinical signs (Al-Natoura *et al*., 2002). Poultry attendants or personnel often serve as disseminators of sporulated oocysts through the attachments of the infective stages to their clothes and shoes (Lawal *et al*., 2008) and coccidiosis has been observed in battery cage birds (Lawal *et al*., 2001). Incubator temperatures kill oocysts so there is no danger of hatchery transmission to chicks (Hofstad *et al*., 1972). New premises are usually contaminated with all species within the first few weeks of occupancy (Hofstad *et al*., 1972). Infection of birds early in their life provides opportunity to establish balance between infection and immunity (Chauhan and Roy, 2007). However, if the infection occurs late in life and exemplified by "new house syndrome", it causes severe disease in older birds, because of lack of prior exposure and hence no immunity (Chauhan and Roy, 2007).

2.5.1 Survival of Oocyts

Moisture, temperature and direct exposure to sunlight all influence the ability of oocysts to sporulate in the external environment, but the interactions of these and other factors, for example mechanical vector such as invertebrates are not well understood. In general, oocysts sporulate more rapidly at higher temperatures and slower at lower temperature; exposures to temperatures less than 10^0C or greater than 50^0C are lethal to unsporulated oocysts (Lawal *et al*., 2008). Between these extremes, the sporulation of oocysts in a field-collected sample is dependent upon at least the following factors: the *Eimeria* species, time, and temperature between collection and arrival of the sample at the laboratory, the medium in which the sample was stored, the amount of molecular oxygen available to the stored oocysts and the concentration of the oocysts in the

sample. Under optimal laboratory conditions, sporulation of oocysts from mammals occur best between 20^0-23^0C and this will vary among vertebrate classes (Duszynski and Wilber, 1997). Once sporulated, oocysts of some species remain viable and infective in 2% aqueous potassium dichromate (kills bacteria and prevents putrifaction) at 4^0-5^0C for up to four years. In their natural external environment, oocysts remain viable and infective from as little as 49 days to up to 86 weeks, dependent upon the species and the interplay of abiotic and biotic environmental parameters.

2.6 Host

2.6.1 Host Susceptibility

Coccidiosis caused by *Eimeria* species affects cattle, deer, sheep, goats, pigs, horses, rabbits, turkeys, ducks and poultry (Molan, 2014). Coccidiosis is a major disease parasite of poultry (Kitandu and Juranova, 2006). The chicken is the only natural host of *Eimeria* species (Hofstad *et al.*, 1972). It causes high mortality in young chicks because most of the *Eimeria* specie affects birds between the ages of 3 and 18 weeks (Hadipour *et al.*, 2013; Shojaei, 2014). It is rarely seen in chicks below 2 weeks of age (Chauhan and Roy, 2007). Day old chicks are susceptible to coccidiosis but out breaks seldom occur at less than 11 days of age (Hofstad *et al*., 1972). Young birds are more susceptible and more readily display signs of disease whereas older chicks are relatively resistant as a result of prior infection (Hafez, 2008).

Susceptibility also depends on several factors such as birds vaccinated against Marek's disease (MD) are more resistant to coccidiosis and MD affected birds are more susceptible to coccidiosis (Chauhan and Roy, 2007). Vitamin A deficiency increases susceptibility to coccidiosis (Chauhan and Roy, 2007). Presence of moisture helps in quick sporulation in coccidial oocysts within 24 to

48 hours and low temperature and dry litter delay sporulation (Chauhan and Roy, 2007). *Eimeria* species are ubiquitous and can survive in infected birds and the environment for long times (Bachaya *et al*., 2012).

2.6.2 Host range

Most Coccidia species are considered to be highly host- specific and only parasitize single host species (oioxenous), although some species in birds and reptiles may parasitize closely related hosts (stenoxenous) and in fish may parasitize unrelated hosts (euryxenous). Many hosts also harbour multiple species of Coccidia which may vary considerably in morphology, developmental cycle, site of infection and pathogenicity (Gyorke *et al*., 2013). In general, the small rapidly developing species are generally the most pathogenic.

2.6.3 Site of Infection

Eimeria species exhibit different tissue and organ specificity (Kitandu and Juranova, 2006). On the basis of affecting organs, the disease is classified as intestinal coccidiosis affecting the small intestine and caecal coccidiosis affecting the caecum (Adhikari *et al.*, 2008). Each *Eimeria* species has a particular predilection site in the chicken digestive tract e.g. *Eimeria tenella* attacks the caecum (Abbas *et al*., 2008). However, in game birds including the quail, the coccidia may parasitize the entire intestinal tract (Gerhold Jr, 2014).

2.6.4 Clinical Signs

Several *Eimeria* species are able to cause clinical signs in infected and unprotected birds, however, subclinical infections are frequently seen (Hafez, 2008). These are often under estimated but mostly result in impaired feed conversion and reduced weight gain (Hafez, 2008).

Coccidiosis is characterized by dysentery, enteritis, emaciation, drooping wing, poor growth, low production (Rehman *et al*., 2010; Awais *et al*., 2012) with high rate of mortality and morbidity (Bachaya *et al*., 2012).

Eimeria tenella causes moderate to severe clinical caecal lesions, sometimes death. Mortality may go as high as 50% (Chauhan and Roy, 2007). Flock mortality up to 20% has occurred within a period of 2-3 days. The presence of dysentery, diarrhoea or soft mucoid faeces will alert the farm owner to a problem, although with less pathogenic species the only sign will be poor growth and impaired feed conversion (Jordan, 1990; Hofstad *et al*., 1972). The birds become depressed, have ruffled feathers, drooping wings, diarrhoea and tend to huddle together, blood loss from caecal lesion and a characteristics odour may be noted before mortality begins (Lillehoj and Trout, 1993; Hofstad *et al.*, 1972). Food and water consumption usually decreases and chicken become emaciated, anaemic and dehydrated (Hafez, 2008). Blood loss may be sufficient as to cause the bird to bleed to death on the fifth to seventh day (Hofstad *et al*., 1972). During the same period, depression in growth rate or weight loss may occur with a maximum loss on the seventh day post infection (Hofstad *et al*, 1972). Laying hen will experience a reduction in rate of egg production (Gerhold Jr, 2014). Weight loss due to dehydration may be rapidly regained during the recovery period but normal weights are never fully regained, during the growing period (Hofstad *et al*., 1972). Coccidiosis is a self limiting disease; most birds recover following survival past day 8. Birds with gangrenous or ruptured caecal pouches may linger for long periods but never fully recover (Hofstad *et al*., 1972). Also infections with certain species of *Eimeria* have been implicated in predisposing birds to necrotic enteritis through lesions that compromise the gut integrity and allow the proliferation of pathogens (Gyorke *et al*., 2013).

2.6.5 Pathogenesis

Pathogenicity is influenced by host genetics, nutritional factors, concurrent diseases, age of the host and species of coccidia (Gerhold Jr, 2014)). The severity also depends on number of sporulated oocysts ingested and the environmental factors (Hafez, 2008). *Eimeria tenella* and *Eimeria necatrix* are the most pathogenic in chickens because schizogony occurs in the lamina propria and Crypts of Lieberkhun of the small intestine and caeca respectively causing extensive haemorrhage (Gerhold Jr, 2014). They also cause high morbidity and mortality in naive birds (Gyorke *et al*., 2013). *Eimeria tenella* primarily infects the caecum and caecal tonsils located at the ileocaecal junction which contain the major source of lymphocytes in the caecum (Yun *et al*., 2000). Developing *E tenella* schizonts have also been reported to be seen in the Bursa of Fabricius (Yun *et al*., 2000). These lymphocytes and other cell types present at the ileocaecal junction are involved in antigen stimulation. Lymphocytes in caecal tonsils consist of 45 to 55% B cells and 35% T cells and are involved in both antibody production and cell mediated immunity (CMI) (Yun *et al*., 2000). Most species develop in the epithelial cells linning the villi (Gerhold Jr, 2014)). Protective immunity usually develops in response to moderate and continuing infection. True age immunity does not occur, but older birds are usually more resistant than young birds because of earlier exposure to infection (Gerhold Jr, 2014). *E acervulina, E maxima* and *E brunetti* cause clinical disease while *E praecox* and *E mitis* considered to be relatively non pathogenic, cause reduced feed conversion efficiency and growth rate (Gyorke *et al*., 2013).

2.6.6 Pathology

The lesions of coccidiosis depend on the degree of inflammation and damage to the intestinal tract. They are also based on sites and vary between species (Jordan, 1990). They include thickness of the intestinal wall, mucoid to blood tinged exudates, petechial haemorrhages, necrosis, haemorrhagic enteritis and profuse bleeding in the caeca (Hafez, 2008). The tissue damage in the intestinal tract may allow secondary colonization by various bacteria such as *Clostridium perfringes* (Hafez, 2008). Infection with *E tenella* also increases the severity of *Histomonas meleagridis* infection in chickens (Hafez, 2008).

Eimeria tenella causes initially blotchy haemorrhagic lesions accompanied by haemorrhage into the caecal lumen and dysentery (Jordan, 1990). The second stage schizonts in their deep sub epithelial position are responsible for the lesions. This phase resolves in 1 or 2 days and the caeca become pale and shrunken with a thickened wall. A core of cellular debris and oocysts forms in the lumen (Jordan, 1990).

Eimeria acervulina is likely to cause lesions in the duodenum with characteristic white irregular linear lesions (Zebra stripling) associated with gamonts and oocysts (Jordan, 1990). In heavy infection, they may coalesce and become less obvious.

Eimeria necatrix causes both white and red focal lesions with ballooning of the intestinal wall and accompanying dysentery (Jordan, 1990). The white lesions are clumps of schizonts (Jordan, 1990). Gametogony and oocysts formation take place in the caeca but without lesions (Jordan, 1990).

Lesions associated with *E maxima* are discrete, focal haemorrhagic lesions which are associated with large gamonts on this species. Soft mucoid salmon pink coloured faeces are typical (Jordan, 1990).

2.6.7 Histopathology

The affected part of the intestine will show large number of parasitic stages such large schizonts in early stages of the disease along with congestion and haemorrhages in the mucosa (Chauhan and Roy, 2007). In the later stages of the disease when it is more likely to be presented for investigation, the histological section will show large number of macro gametocytes and microgametocyte with large number of microgametes within it and some mature oocyts in the epithelial cells (Chauhan and Roy, 2007). Congestion of the mucosa largely subsides and there's infiltration of lymphocytes and some other mononuclear cells in the mucosa. Theirs is marked denudation of mucosal epithelium at this stage (Chauhan and Roy, 2007).

Intestinal coccidiosis may be confused with necrotic enteritis, haemorrhagic enteritis or other enteric diseases (Hafez, 2008). Caecal coccidiosis may be confused with histomoniasis and salmonellosis due to their similar lesions (Hafez, 2008).

2.7.0 Epidemiology

Economic losses due to coccidiosis are significant being estimated at more than 3 billion US dollars annually in the world (Gyorke *et al*., 2013), and the economic importance of subclinical coccidiosis varies with the composition of clinical coccidial populations (Haug *et al*., 2008). Due to higher stocking densities and intensive husbandry practices its incidence is being increased in poultry (Bachaya *et al*., 2012). Haug *et al*. (2008) reports that the economic impact of subclinical infection is considerable and is the main concern keeping commercial view in

consideration as the subclinical form of disease shows negative effect on the performance of infected birds and leads to impaired feed conversion which in turn causes great economic losses to poultry industry throughout the world. He also reported a substantial geographical differences in both prevalence and infection levels.

Amare *et al*. (2012) conducted a study to evaluate the prevalence of coccidiosis, identify the prevalent species of *Eimeria* and to assess the predisposing factors. He observed an overall prevalence of 25.24% of clinical coccidiosis assessed from which was recorded prevalence rates of 22.3% and 35.3% respectively for the White leghorn (WLH) grower chickens and parent stocks of the WLH and Rhode Island Red (RIR) breeds. The prevalence of clinical coccidiosis was significantly higher in adults than the growers ($P<0.05$). However, no statistically significant difference was observed between sexes and breeds. Species identified were *E tenella, E brunetti, E. necatrix, E acervulina* and *E maxima*.

Sun *et al*. (2009) examined the faecal samples of chicken from poultry farms for *Eimeria* species. The infection rate of identified *Eimeria* in these farms were 90%, 88%, 72%, 68%, 60%, 26% and 8% for *E tenella, E praecox, E acervulina, E maxima E mitis, E necatrix* and *E brunetti* respectively.

In China, the prevalence of *Eimeria tenella* is the highest with it presenting an explosive appearance of caecal coccidiosis in the acute phase and where the mortality rate can reach up to 50-80% in a few days. In recent years, the prevalence of intestinal coccidiosis is increasing especially *E necatrix* infection with increase in South China.

Awais *et al*. (2012) reported on the prevalence of coccidiosis in industrial broiler chickens in Pakistan. *Eimeria tenella* had the highest prevalence (27.04%) followed by *E. maxima* (22.42%),

E acervulina (19.89%) and *E necatrix* (4.02%). The prevalence of the disease was significantly higher in the autum (60.02) followed by summer (47.42), spring (36.92) and winter (29.89).

In Nepal, Adhikari *et al*. (2008) reported that the highest prevalence rate month wise for coccidial infection of *E tenella* (50%) was observed in March and the lowest (10%) in the months of April and September. Season wise the highest prevalence rate (33%) was in the summer and spring followed by 14% in the autumn. In layer birds, highest prevalence rate age wise (48%) was in 31-45 days age group and the least (6%) in 0-15 days age group of layers (Adhikari *et al*., 2008).

In Iran, Nematollahi *et al*. (2009) reported an overall prevalence of coccidiosis in broiler chicken to be 55.96% and reported that the prevalence of infection increased with the age of chickens, with chickens 5 weeks of age showing the highest prevalence of infection.

While coccidiosis has been demonstrated to cause production losses affecting profitability of individual farms in Europe and the United State (Fornace *et al*., 2013), the occurrence and economic relevance of coccidiosis in poultry farms in Africa remain largely unknown (Fornace *et al*., 2013). Within Africa and other areas of the developing world, increasing human population, income levels and urbanization have led to an increase in livestock production often referred to as the "Livestock revolution" (Thornton, 2010). Small scale intensive livestock industries have emerged in peri-urban areas of many developing countries, mainly based on monogastric species with specialized breeds fed on concentrate feeds. With an annual growth rate of 2.1%, poultry is one of the fastest growing livestock sectors, providing an additional source of income and an affordable and accessible source of protein to many poor households (Otte *et al*., 2008). Over 85% of rural households in sub-Saharan Africa keep poultry with

smaller scale commercial farms (less than 2000 birds per batch) believed to play a critical role in providing households with economic opportunities and enough food security (Fornace *et al*., 2013). As women frequently manage household poultry production in developing countries, income from poultry production in developing countries can additionally be a method to support women's livelihood (Fornace *et al*., 2013). While traditional (backyard) poultry production systems consist of few free ranging dual purpose indigenous birds kept mainly for household use with few inputs and high mortality, small scale commercial family raise poultry primarily as an income generating activity keeping broilers or layers in semi-intensive conditions with food and water usually provided (Fornace *et al*., 2013). These emerging small scale systems have been identified for development and an important source of income for African households (Fornace *et al*., 2013). Adoption of intensive farming methods with limited biosecurity may create an environment conducive to diseases. Disease challenges faced by these farms are poorly characterized and have important implication for households' poverty and nutritional status (Fornace *et al*., 2013).

In Ethopia, poultry coccidiosis is endemic in various parts of the country and affects mainly young growing birds (Meskerem and Boonkaewwan, 2013). Mersha *et al*. (2009) reported a prevalence of clinical coccidiosis from 3 commercial broiler farms in Central Ethiopa to be 37.5, 43.33 and 50% respectively and the *Eimeria* identified were *E tenella, E necatrix, E acervulina*. Losses due to mortality following a severe outbreak may be devastating and incidences rate as high as 80% were observed to occur in an outbreak in Ethiopia. Fornace *et al.* (2013) reported the detection of coccidiosis in farms in Ghana, Tanzania and Zambia. Reliance on imported day old chicks can introduce different *Eimeria* species and strains into these environments as exemplified by the presence of OTU's (Operational Taxonomic Units which are three genetic

variants) previously only identified in Australia (Fornace *et al*., 2013). Dakpogan and Salifou (2013) reported rate of 36.6% of prevalence of litter based high stocking density layer rearing system Benin which is a proof of the endemicity of the disease in the country.

Epidemiological studies in Nigeria have established the economic importance of coccidiosis as a major parasitic disease of poultry (Abdu, 2007; Majaro, 2001), though data on avian coccidiosis in north western Nigeria are scarce and not up to date (Jatau *et al*., 2012). Such information is very important for proper diagnosis and control of the disease and for the selection of appropriate anti coccidial drugs (Sun *et al*., 2009; Lee *et al*., 2010). The disease is said to occur throughout the year in Northern Nigeria but with higher prevalence rates from May to September (Abdu, 2007). Outbreaks of up to 50% mortality have been reported in commercial poultry in Zaria, Nigeria (Abdu *et al*., 2008). Majaro (2001) reported and described the seven *Eimeria* species in exotic commercial broiler chickens. Muazu *et al*. (2008) investigated the prevalence of coccidiosis and identification of species in Vom, Plateau State from nine different poultry farms during the months of April - June 2008. The prevalence of coccidial infection among adult birds was 36.7% while the younger birds were 52.9%. The species of *Eimeria* identified include *E tenella, E maxima, E necatrix* and *E acervulina*.

2.8.0 Diagnosis

Coccidiosis is often extremely difficult to diagnose (Hafez, 2008). Diagnosis of *Eimeria* infections and differentiation between species is usually attempted by consideration of clinical signs in the host and the biological features of the parasite (Hamidinejat *et al*., 2010; Patra *et al*., 2010). The latter include prepatent period, site of development within the intestine and the morphological appearance of the oocysts in the faeces and endogenous stages in the intestinal or

caecal mucosa as well as sporulation time (Hafez, 2008; Patra *et al*., 2010; Gyorke *et al*., 2013). Analysis of these characteristics is labour intensive for diagnosis and does not provide accurate data for identification of *Eimeria* species (Hamidinejat *et al*., 2010). Diagnostic laboratories are increasingly utilizing DNA based technologies for the specific identification of the parasite (Morgan *et al*., 2009; Hamidinejat *et al,* 2010). The presence of the parasites may be confirmed by post mortem examination of the host or faecal examination (Patra *et al*., 2010). *Eimeria tenella* and *E necatrix* have unmistakable lesions and are pathognomonic (Gerhold Jr, 2014). Mixed infections are commonly found in field conditions which pose a problem for precise discrimination of species using classical methods (Gyorke *et al*., 2013). A diagnosis of clinical coccidiosis is warranted if oocysts, merozoites or schizonts are seen microscopically and if the lesions are severe (Gerhold Jr, 2014). Although some clinical eimerial infections may be unimportant (Gerhold Jr, 2014) they can cause considerable depression in feed efficiency and egg production, which accounts for 70% of the total coccidiosis diseases control cost (Haug *et al*., 2008; Dakpogan and Salifou, 2013).

Polymerase Chain Reaction (PCR) based assays have proved to be effective for the identification of all seven species of *Eimeria* in chickens (Gyorke *et al*., 2013). A number of approaches have also proved to be both specific and highly sensitive for analysis, either of parasites grown *in vitro* or present in tissue samples and clinical materials (Beate *et al*., 1998; Su *et al*., 2003; Kawahara *et al*., 2008; Patra *et al*., 2010). An attractive genomic DNA target for PCR analysis is the Internal Transcribed Spacer 1 (ITS-1) and Internal Transcribed Spacer 2 (ITS-2) gene of ribosomal DNA (rDNA) (Haug *et al*., 2007; Patra *et al*., 2010 Gyorke *et al*., 2013). This spacer separates the 3 ends of the 16S-like ribosomal RNA gene from the 5 end of the 5.8 S rRNA gene within individual rDNA transcription units (Patra *et al*., 2010). Due to its heterogenecity in both

sequence length and base composition, the ITS-1 lends itself perfectly for the design of specific primers and as part of the rDNA transcription unit (Patra *et al*., 2010). It is also a member of a multiple copy gene family and thus provides large numbers of potential PCR targets (Patra *et al*., 2010). The target regions are small subunit r RNA (Gyorke *et al*., 2013), 5 S rRNA (Patra *et al*., 2010), first and second Internal Transcribed Spacer (ITS-1 and ITS-2) of nuclear rDNA (Haug *et al*., 2007) and sequence characterized amplified region (SCAR) derived from Random Amplified Polymorphic DNA (RAPD) (Gyorke *et al*., 2013).

Examination of the faecal contents or scrapping from affected mucosa of the intestine shows oval, thick walled oocysts, large round multi karyotic schizonts and gametocytes in significant numbers (Chauhan and Roy, 2007).

2.9.0 Treatment

In the past, it was realized that eradication of coccidiosis is not realistic and that hygienic measures alone are not able to prevent infections (Hafez, 2008; Gerhold Jr, 2014). However, it is advisable that if an outbreak of coccidiosis occurs, treatment via the drinking water should start as soon as possible (Hafez, 2008). The most commonly used anticoccidials (Sulphonamide, Amprolium and Toltrazuril) and increased levels of vitamin A and K are sometimes used in rations to improve rate of recovery and prevent secondary bacterial infection (Hafez, 2008; Gerhold Jr, 2014).

Outbreaks can generally be controlled by management practices based on improving hygiene, reducing overcrowding, removing contaminated litter and isolation of affected birds (Chauhan and Roy, 2007).

2.9.1 Prevention and control

Prevention and control of coccidiosis is based on chemotherapy, using anti coccidial drugs and/or vaccines along with hygienic measures and improved farm management (Hafez, 2008). Currently coccidiosis is controlled mainly with drugs (Xu *et al*., 2013). The effective use of anticoccidial drugs over the past 50 years has played a major role in the growth of the poultry industry and has allowed the increased availability of high quality and affordable poultry products to the consumers (Allen and Fetterer, 2002; Kitandu and Juranova, 2006). Numerous products were introduced, many of which are available and in use today (Kitandu and Juranova, 2006; Hafez, 2008). However, increasing concerns about rising levels of drug resistance has gained prominence (Chapman, 1997).

2.9.2 Anticoccidial Drugs

The use of anticoccidial drugs became the treatment of choice in the poultry industry as large volumes of manufacturing methods and market competition reduced the cost of treatment (Price and Barta, 2010). The use of anti coccidial drugs allowed for more birds to be reared in a single house (McDougald, 2003; Hafez, 2008). However, these products also prevented treated birds from building up any natural immunity and were not effective enough to kill all Coccidia in a bird (Hafez, 2008). The result was that the surviving Coccidia quickly became resistant to the products resulting in severe outbreaks of the disease (Hafez, 2008). Legislation demands that most anti coccidial drugs must be removed from the medicated feed approximately a week before marketing and for effectiveness (McDougald, 2003; Price and Barta, 2010). Legislation was introduced because of concern for drug residues in food which may be toxic to man (Vermeulen *et al*., 2001; Price and Barta, 2010). However, the withdrawal of drugs could

increase the susceptibility of birds to infection because they may not have acquired protective immunity while on treatment with anticoccidial drugs (Price and Barta, 2010). Prophylactic treatment with anticoccidial drug has led to the emergence of drug resistant strains due to prolonged use in the field (McDougald, 2003; Price and Barta, 2010). To combat this, management programmes are designed to prevent this from happening which results in better gut health and feed utilization by birds (Hafez, 2008). Using a drug rotation (with constant monitoring of the oocyst in the faeces and litter) or shuttle programme seems to be of a great value (Hafez, 2008; Price and Barta, 2010). Rotation involves changing the product in use every 4-6 months (Hafez, 2008) and the alternative to a rotation programme is a continuous programme where the same products are used until a problem develops or until a new product is introduced on the market (Hafez, 2008). Rotation is only possible if drugs with different modes of action follow each other (Hafez, 2008). A shuttle programme uses two or more products during the grow-out of a flock (Hafez, 2008; Gerhold Jr, 2014). The principle is to use the drug most suited to each phase of the grow-out, so that one drug is used for the starter period, while another is used during the grower and finisher phase (Hafez, 2008). The drug withdrawal period is a very important consideration for treatment in finisher feeds. A switch system can also be used where the anticoccidial agent is changed at each restocking within an operation. Lee *et al*. (2009) discovered that careful use of the shuttle rotation programme does not fully prevent drug resistance but increases the useful longevity of the drug. An outbreak of coccidiosis is often an indication of immunosuppression (Hafez, 2008). Concurrent infection with immunosuppressive diseases such as Marek's disease may interfere with development of immunity to coccidiosis and infectious bursal disease may exacerbate coccidiosis, placing a heavier burden on anticoccidial drug treatment (Hafez, 2008).

The effects of anticoccidial drugs may be coccidiostatic in nature in which growth of intracellular Coccidia is arrested but development may continue after drug withdrawal (Gerhold Jr, 2014) or coccidiocidal in which coccidia are killed during their development (Gerhold Jr, 2014). Most anticoccidiasl currently used in poultry production are coccidiocidal (Gerhold, Jr, 2014). Anticoccidial drugs may be used in feed in preventive doses (Chauhan and Roy 2007). This causes natural development of immunity to coccidiosis and may proceed during the use of anticoccidials in the feed (Gerhold Jr, 2014). However, this may be of little consequence on the production of broilers during a short grow-out period of 37-44 days (Gerhold, Jr, 2014). Natural immunity is important in replacement layer pullets because they are likely to be exposed to coccidial infections for extended periods after termination of anticoccidial drugs (Gerhold, Jr, 2014). Anticoccidial programmes for layer and breeder flocks are intended to allow immunizing infection while guarding against acute out breaks (Gerhold, Jr. 2014). Anticoccidials are commonly withdrawn from broilers 3-7 days before slaughter to meet regulatory requirements and to reduce production costs (Gerhold Jr, 2014). Due to the fact that broilers have varying susceptibility to infection at this point, the risk of coccidiosis out breaks is increased with longer withdrawal period. Turkeys are given a preventive anticoccidial for confinement –reared birds up to 8-10 weeks of age. Older birds are considered less susceptible to outbreaks (Gerhold Jr, 2014).

A major enhancement in coccidiosis control occurred in the 1970's with the introduction of Monensin as the first ionophore coccidiostat (Hafez, 2008). Introduction of ionophores changed the ability to control coccidiosis, an impact that remains to this day (Shirley and Chapman, 2005). The effectiveness of ionophore coccidiostats lies in the fact that while they kill the majority of the invading parasites, they permit a small leakage of coccidia enabling a degree of host immunity to develop (Hafez, 2008). Therefore resistance to ionophore develops very slowly

with a tendency to increased levels of tolerance (Hafez, 2008). But even monesin and some of the new ionophorous antibiotic, anticoccidial drugs used in the field have had inevitably drug resistant parasites emerged (Price and Barta, 2010).

Since 1970's coccidiostats have been regulated under the feed additives directive 524/70/EEC (EEC, 1970; 2004), which has now been replaced by Regulation No 1831/2003 IEC (EC, 2003; 2007). As such they have not been subject to veterinary prescription status since they are required routinely in the feed of commercial broilers and turkeys (Hafez, 2008). Currently several types of anticoccidial drugs are available including synthetic compounds, quinolones and certain ionophore antibiotics (Hafez, 2008). In recent years however, few new drugs have been introduced. All types of drugs used for coccidiosis control are unique: their mode of action, the way parasites are killed or arrested and the effects of the drugs on the growth and performance of the bird. Very few drugs are equally efficacious against all *Eimeria* species (McDougald, 2003).

The modes of action of anticoccidial drugs are poorly understood. Some that are better known are described below as knowledge of mode of action is important in understanding toxicity and adverse effects (Gerhold Jr, 2014).

Anticoccidial drugs can be classified as either synthetic or non synthetic (natural) compounds:

2.9.2.1 Synthetic drugs (chemicals):

These were the first to be introduced as anticoccidials (Kitandu and Juranova, 2006) and have specific modes of action against parasite metabolism. Examples include: amprolium, clopidol decoquinate and halofuginone and sulphonamide anti bacterials. Sulphonamides and related drugs compete for the incorporation of Par amino benzoic acid (PABA) and metabolism of folic acid. Sulphonamides also act on the developing schizonts and also on the sexual stages (Kitandu

and Juranova, 2006). Amprolium compete for absorption of thiamine by the parasite.Quinoxaline and clopidol inhibit energy metabolism in the cytochrome system of coccidia (Kitandu and Juranova, 2006). The quinolones arrest or kill the sporozoite or early trophozoite while nicarbazin, robenidine and zoalene destroy the first and second generation schizonts.

2.9.2.2 Non synthetic (natural) anticoccidial drug:

Polyether ionophores such as monensin, lasalocid, salinomycin, narasin maduramicin which act through general mechanisms of altering ion transport and disrupting osmotic balance (Allen and Fetterer, 2002). The ionophores kill coccidia by interfering with the balance of important ions such as sodium and potassium. The host cells are able to manage these ions in the presence of ionophores but the parasite cannot (Kitandu and Juranova, 2006). The ionophores are now the mainstay of coccidiosis control (Allen and Fetterer, 2002; Kitandu and Juranova, 2006).

2.9.3 Specific anticoccidial Drugs:

2.9.3.1 Amprolium: Amprolium is an antagonist of thiamine (vitamin B1) (Gerhold Jr, 2014). Rapidly dividing coccidia have a high requirement for thiamine (Gerhold Jr, 2014). Due to the poor activity of amprolium against some *Eimeria* spp, its spectrum has been extended by using it in mixtures with the folic acid antagonists, ethopabate and sulfaquinoxaline (Gerhold Jr, 2014). The primary use of Amprolium today is for water treatment during clinical out breaks of coccidiosis (Gerhold Jr, 2014).

2.9.3.2 Diaclazuril and Toltrazuril: These are recent anticoccidial drugs used in the control of coccidiosis (Gerhold, Jr, 2014). The use of Toltrazuril as the sole anticoccidial for two consecutive days in the drinking water between days 10 and 14 would be the best time for good coccidiosis control (Muir and Aggrey 2003). It was reported that Toltrazuril is active against all intracellular developmental stages including those of schizogony and

gametogony and does not interfere with the development of natural immunity (Kitandu and Juranova, 2006). Diaclazuril is used mainly for prevention at 1ppm in the feed while Toltrazuril is used primarily for treatment in the water.

2.9.3.3 Clopidol and Quinolones (Decoquinate, Methylbenzoquate): They are coccidiostatic against early development of *Eimeria* species by inhibiting mitochondrial energy production (Gerhold Jr, 2006). Clopidol and quinolones have a broad species spectrum and are sometimes mixed together for synergism. However, resistance may develop rapidly during extended use (Gerhold Jr, 2014).

2.9.3.4 Folic acid antagonists: These include the sulphonamides, 2, 4- diamino pyrimidines and ethopabate (Gerhold, Jr, 2014). These compounds are structural antagonists of folic acid or of paramino benzoic acid (PABA) which is a precursor of folic acid (Gerhold Jr, 2014). Coccidia rapidly synthesize nucleic acid, accounting for activity of PABA antagonists (Gerhold Jr, 2014). Although resistance to anti folate compounds is widespread, they are commonly used for water treatment when clinical signs are already evident (Gerhold Jr, 2014). Diaveridine, Ormetoprim and pyrimethamine are active against the protozoan enzyme dihydrofolate reductase. They have synergistic activity with sulfonamides and are often used in mixtures with these compounds (Gerhold Jr, 2014).

2.9.3.5 Halofuginone hydrobromide: This is related to the antimalarial drug febrifuginone and is effective against asexual stages of most species of *Eimeria*. It has both coccidiostatic and coccidiocidal effects but coccidia may become resistant after extended exposure (Gerhold Jr, 2014).

2.9.3.6 Polyether ionophores: Since 1971, the preferred drugs for coccidiosis prevention have been ionophore antibiotics (Kitandu and Juranova, 2006). The ionophores (Monensin, Salinomycin, Lasalocid, Narasin, Maduramicin and Semduramicin) form complexes with various ions, principally sodium, potassium and calcium and transport these into and through biologic membranes (Gerhold Jr, 2014). The ionophores affect both extra and intracellular stages of the parasite, especially during early asexual stages of parasite development (Gerhold, Jr, 2014). Salinomycin, monensin, lasalocid, maduramicin and semduramicin remain useful agents to activate sufficient control despite resistance being common (Kitandu and Juranova, 2006). However they cannot be used in situations of heavy parasite challenge (Kitandu and Juranova, 2006). It has also been demonstrated that some ionophores can be used in combination with live virulent vaccines. The advantage of such ionophores is that they prevent infection during the first 3-4 weeks of age when immunity is not developed (Kitandu and Juranova, 2006). Some ionophores may depress feed consumption when the dosage is above recommended levels but these products remain the most important class of anticoccidials (Gerhold, Jr, 2014).

2.9.3.7 Nicarbazin was the first product to have truly broad spectrum activity and has been in common use since 1955 (Gerhold Jr, 2014). The mode of action though not completely understood is thought to be via inhibition of succinate linked nicotamide adenine dinucleotide reduction and the energy dependent transhydrogenase, and the accumulation of calcium in the presence of ATP. Nicarbazin is toxic for layers causing mottling of egg yolks, decreased egg production and blanching of brown egg shells. A 4-day withdrawal period is required in broilers and medicated birds are at risk of increased heat stress in hot weather (Gerhold Jr, 2014).

2.9.3.8 Nitrobenzamides (e.g. Dinitolmide exert their greatest coccidiostatic activity against the asexual stages. Efficiency is limited to *E tenella* and *E necatrix* unless combined with other products (Gerhold Jr, 2015).

2.9.3.9 Robenidine is a guanidine compound that allows initial intracellular development of coccidia but prevents the formation of mature schizonts. It is coccidiostatic when given short term and coccidiocidal long term. Drug existence may develop during use. A 5 -day withdrawal period is needed to eliminate untoward flavour caused by residues in poultry meat (Gerhold Jr, 2014).

2.9.3.10 Roxarsone is an organic arsenical compound which has significant activity against *E .tenella* and is used in combination with ionophores to improve control and a withdrawal period is required (Gerhold Jr, 2014).

The chemical classes of commoly used anticoccidials and the stages of the *Eimeria* species the drugs act on listed in table 2.3.

Table 2.3: Chemical classes of commonly used anticoccidials

Class of drug	Examples of Drugs	Coccidial life cycle affected
Ionophores	Monensin, lasalocid, narasin1q2wasz, maduramicin and semduramicin	Trophozoite/sporozoite
Sulphonamides	Sulphaquinoxaline (+ DHFR inhibitors)	Second and later schizont
Quinolones	Decoquinate	Sporozoite
Thiamine mimics	Amprolium, Halofuginone	First generation schizont
Guanidine	Robenidine Nicarbazin	Asexual stages
	Diaclazuril and Toltrazuril	Multiple stages

Cited by Kitandu and Juranova (2006)

2.9.4.0 Vaccines

Coccidiosis is one of the major challenge of poultry industry which is compounded by the problem of anticoccidial drug resistance, lack of new anticoccidial products, susceptibility of turkeys to ionophore toxicity and consumer demanding decrease in the use of antibiotics in animal feed (Hafez, 2008). There is an urgent need to seek alternative strategies to control coccidiosis which has made the use of vaccines more attractive (Allen and Fetterer, 2002; Kitandu and Juranova, 2006; Hafez 2008).

Avian Coccidia are highly immunogenic and primary infections can stimulate solid immunity to homologous challenges (Allen and Fetterer 2002; Price and Barta, 2010). Identification of

parasite life cycle stages and development- specific antigens inducing protective immunity is a critical step in recombinant protein vaccine development (Min *et al*., 2004). In the case of *Eimeria*, a recombinant form of both parasite surface antigens and internal antigens has been investigated as vaccine candidates (Min *et al*., 2004). Sporozoites are the preferred parasitic forms and cell surface antigens are logical components of vaccines because of their direct role in host- parasite relationship (Min *et al*., 2004). Recently attempts to immunise birds against coccidiosis has been made which showed that *E adenoides* of turkeys can protect chickens against *E tenella* if repeated innoculations with the oocysts of *E adenoides* are given (Chauhan and Roy, 2007). New generations of safe, live attenuated vaccines are now on the verge of being commercially available (Chauhan and Roy, 2007). Some virulent coccidial oocyst vaccines are now available such as Coccivac® and Immucox® (Chauhan and Roy, 2007). A major disadvantage of vaccines made with virulent type of coccidia is that if the vaccine dose is not carefully controlled then severe disease may occur in those birds which do not ingest vaccinal oocyts thus remain highly susceptible. New live vaccines such as Paracox® are safe though live because they have genetically low reproductive index, hence they cannot produce pathogenic numbers required to produce disease.

Attempts to produce recombinant vaccines are being made (Chauhan and Roy, 2007). This attempt includes microorganisms having recombinant genes which during the course of their replication produce coccidial antigens (Chauhan and Roy, 2007). Such antigens injected into birds may produce anticoccidial immunity (Chauhan and Roy, 2007). Such successful attempt has been made in the case of *E tenella* and it has also been demonstrated that crude extracts of parasites can be used as vaccines (Chauhan and Roy, 2007). A new live attenuated vaccine

having *E acervulina, E maxima*, *E mitis* and *E tenella* was found successful in the UK (Chauhan and Roy, 2007).

Although vaccines played a role in coccidiosis control for a long time, their applications still remain very limited with consideration for the safety, costs and demands for high techniques by the farmers or veterinarians (Xu *et al*., 2013).

A few anticoccidial products have been tested as potential alternatives to drug or vaccines. These include probiotics, herbal extracts and antibodies (Xu *et al*., 2013). Among these, passive immunization with antibody is a highly attractive approach due to its high specificity, even though this procedure is invasive as chickens are usually sacrificed in order to obtain sufficient antibodies (Xu *et al*., 2013). Chicken egg yolk immunoglobulin (IgY) is immunoglobulin transferred from serum of hens to the egg yolk which offers passive immunity to newly hatched chicks (Xu *et al*., 2013). This method has received attention due to its non invasive and cost affective characteristics and has been used to successfully control infectious diseases in poultry (Xu *et al*., 2013). Recently a commercial hyper immune egg yolk IgY powder named Supracox against 3 species of *Eimeria* was demonstrated to provide certain protection against experimental infections of *E tenella*, *E maxima* and *E acervulina* (Lee *et al*., 2009, Xu *et al.*, 2013).

It is known that when chickens are infected with low number of *Eimeria* species, protective immunity is induced after two or three consecutive infections (Kitandu and Juranova, 2006). Live vaccines have been used mostly in breeder stocks and to a lesser extent in commercial broilers and replacement hens.

2.9.4.1 Live vaccines used in the control of the coccidiosis

Live vaccines that are vused to control coccidiosis in [poultry include the following:

i. **Live virulent strain:** Comprise of a variable number of wild type strains incorporated and depends on their formulation and field of application. For broiler breeders, upto 8 *Eimeria* species are included in these products e.g. (Coccivac® D immuncox® C2). For the broiler industry, this number is restricted up-to four species (Coccivac® B, immuncox® C1). The advantage of live virulent vaccines is that they can provide equal or superior performance compared with prophylactic medication when given as gel form on day 1 of age because this method ensures the synchronous exposure of all birds to small uniform number of oocysts (Kitandu and Juranova, 2006).

ii. **Live attenuated strain:** This type of vaccine can be obtained by repeated selection for early maturation (precociousness) or serial passage through embroyonated eggs (TA lines). Examples are Paracox (precocious strain) and Livacox ® (precocious and TA strains) (Kitandu and Juranova, 2006). The advantage of live attenuated vaccines is that they have low productive potentials resulting in development of optimal immunity with minimal tissue damage and cannot induce coccidial dry resistance (Kitandu and Juranova, 2006).

iii. **Live, tolerant to ionophores:** There is a new development that has made live *Eimeria* strains relatively tolerant to ionophores. An experimental vaccine comprising of a strain of *E. acervulina* and a strain from *E. maxima*, both of which were partially tolerant to Salinomycin, has been used in USA; so also is a live vaccine that can be used with different ionophores (Nobilis® Cox ATM) (Kitandu and Juranova, 2006).

2.9.5.0 Alternative control of coccidiosis

Different types of substances have been investigated in search of alternative control for coccidiosis (Allen and Fetterer, 2002). Natural medicinal products as feed supplements have

been widely used as growth and health promoters in farm animals (Kitandu and Juranova, 2006). A current estimation of the number of immune active natural medicinal products ranges between 200 and 300 and most products originate from plants and fungi (Li, 2001). The immuno active components of these plants and fungi include polysaccharides, glycosides, alkaloids, volatile oils, and organic acids of which polysaccharides are considered to be the most important (Li, 2001). Polysaccharides may act as immune enhancers or immuno modulators and these components may display antibacterial activity (Kitandu and Juranova, 2006).

Alternative control of coccidiosis including Nutritional and Probiotics (immune modulators) or Natural feed additives are described below:

I. Fat diet:

Diets containing high concentration of fatty acids (n-3 FA) docosa hexaenoic acid, eicosa pentaenoic acid and linolenic acids) such as fish oils, flax seed oil, and whole flax seed when added to starter ration and fed to chicks from 1 day of age effectively reduced lesions resulting from challenge infection with *E tenella* (Allen and Fetterer, 2002; Kitandu and Juranova, 2006) but not *E maxima* (Allen *et al*., 1997). The fish oil and flax seed oil diets significantly reduced the degree of parasitization by and development of *E tenella* (Allen *et al*., 1998) and caused ultra structural degradation of both asexual and sexual stages (Kitandu and Juranova, 2006).

II. Vitamin A:

It is known that nutrition plays a role in the development of chicken immune system (Kitandu and Juranova, 2006). Essential nutrients such as vitamins may affect both humoral and cell mediated immune responses. Vitamin A known for its role in the differentiation of

epithelial cells is essential for maintaining the integrity of mucosal surfaces (Kitandu and Juranova, 2006). Deficiency of vitamin A increases host susceptibility to enteric diseases like coccidiosis (Dalloul *et al*., 2002). Feed supplementation with antioxidants such as ɤ-tocopherol (8 ppm), found plentiful in seed oils such as wheat, corn and soy bean and the spice turmeric (1%) as well as its main medicinal component curcumin (0.05%) appear effective in reducing upper and mid-small intestinal infections caused by *E. acervulina* and *E maxima*. They are not beneficial for *E tenella* infection. (Allen and Fetterer, 2002).

III. Betaine:

Betaine is another dietary supplement, a naturally occurring amino acid derivative that has been investigated as potential enhancing agent against coccidiosis (Kitandu and Juranova, 2006). The osmo protectant betaine (from sugar beets) has long been known to have beneficial effects on livestock growths and performance (Allen and Fetterer, 2002). Studies have shown differential effects on the rate of body weight gain in chickens infected with different *Eimeria* species where it was effective only during *E maxima* infection but not during *E acervulina* or *E tenella* infections (Kitandu and Juranova, 2006). When given in Salinomycin treated chickens, betaine significantly reduced invasion by *E acervulina* and *E tenella* (Kitandu and Juranova, 2006).

IV. Probiotics:

Probiotic supplementation of the intestinal microflora has been shown to enhance gut defensive mechanisms in poultry (La Ragione *et al*., 2004; Kitandu and Juranova, 2006). The development and the use of probiotics for animals including poultry is based on the knowledge that the gut flora is involved in resistance to enteric infections where it has been shown to be involved in protection against a variety of pathogens including *Escherichia coli*,

Salmonella spp, *Campylobacter jejuni* and more recently *Eimeria* spp (Kitandu and Juranova, 2006). Several studies have demonstrated that a Lactobacillus based probiotic stimulated local immune system of broiler chicken and improved resistance to *E acervulina.* Reports were that probiotic fed chickens shed fewer oocysts than did chickens without probiotics even in vitamin A deficient birds (Kitandu and Juronova, 2006).

V. CPG Oligodeoxynucleotide (ODN):

ODNs have been shown to play a role in host defence both by stimulating T cells and by inducing cytokines or enhancing innate immunity (Kitandu and Juranova, 2006). Studies by Dalloul *et al*. (2004) and Xie *et al.* (2003) identified CpG sequences that activate chicken innate immunity and enhance protective immune response against *Salmonella* spp and coccidia (Kitandu and Juranova, 2006).

V. Herbs:

Artemisinin is a Chinese herb isolated from *Artemisia annua* which is a naturally occurring endoperoxide with antimalarial properties. It has been found effective in reducing oocyst output from both *E acervulina* and *E tenella* infections when fed at levels of 8.5 and 17ppm in starter diets (Kitandu and Juranova, 2006). Most recently, extracts from 15 Asian herbs were tested for anticoccidial activity against *E tenella* and *Sophora flavescens* (Allen and Fetterer, 2002; Kitandu and Juranova 2006).

2.10.0 The Plant *Khaya senegalensis*

The plant *Khaya senegalensis* belongs to the Family *Meliaceace*. The common English name of the plant is dry zone Mahogany (Burkill, 1985). The plant is also called “Madaci”, “Oganwo”, “Ono”, “Daalehi” or “Kahi” and “Wuchi” in Hausa, Yoruba, Igbo, Fulfulde and Nupe languages

respectively in Nigeria (Mann *et al*., 2003). Trade names of the plant include Lagos mahogany and Benin wood (Burkill, 1985).

2.10.1 Morphological Description

The tree is about 30 metres (m) tall (Burkill, 1985). It has a dense crown and short bole covered with dark grey scaly bark (Mann *et al*., 2003). The leaves are imparipinnate with 3-7 pairs of leaflets that are oblong 6-12 centimeters (cm) long and 2-5cm broad. The apex is rounded or shortly acuminated and glabrous with lateral nerves of 8-16 pairs (Mann *et al*., 2003). Inflorescence consists of flowers that have pale-green sepals, cream petals and staminal tub with bright red disc beneath the ovary. Fruits are capsular and dehiscent with 4-5 valves of 4-6cm diameter that open to expose orbicular winged seeds tightly arranged inside. The flowering period is between January and April and the plant is widely distributed in the Savanna region (Mann *et al*., 2003). The tree is commonly grown in the Sudan-guinean zone as a shade and avenue tree (Burkill, 1985). Sap wood is nearly white or tinged purple while Heart wood is red-brown also tinged purple, the two merging together without marked separation. The wood is perishable but is easy to treat with preservative. The Heart wood when brown is relatively durable and is resistant to treatment (Burkill, 1985). When sawn, there is a slight aromatic odour due to presence of vessels containing resin. This is said to confer an ability of the wood to keep sound in water (Burkill, 1985).

2.10.2 Nutritional value and compounds isolated from the bark and leaves

The nutritional value of *Khaya senegalensis* leaves are 12% crude protein, 43% neutral detergent fibres and 17% ash in dry matter basis (Awohouedji *et al*., 2013). Several active compounds have been isolated from *Khaya senegalensis* bark and leaves (Makut *et al*., 2008; Otu *et al*.,

2009) including saponins, tannins, alkanoids, glycosides, steroids, calcedrin, terpenoids and flavonoids. Some limnoids have also been isolated from *Khaya senegalensis* bark and leaves (Nakatani *et al*., 2002; Yaun *et al*., 2013. Limnoids are highly oxygenated terpenoids and they have also been described as mordified triterpene having a 4,4,8 trimethyl-17 furanyl steroid skeleton. Arrangement of subgroups and ring structures within this basic building block provide a host of characteristics that have generated a lot of interest s. These characteristics include insecticidal, insect growth regulation, insect antifeedant and and have a range of pharmacological activities and medicinal effects to humans and animals such as antibacterial, antifungal, antimalarial, anti cancer and anti viral effects (Sunhwa *et al.,* 2014). Two new compounds Khaya seneganin I and 2α,3α,16β-trihdroxy-20-acetoxy-20(*R*)-pregnane along with six known compounds , 2α,3α20-trihydroxy-16β-acetoxy-20(*R*)-pregnane, 2α,3β-dihdroxypregnane-16-one-2β,19-hemiketal, (+)-catechin, ivorenolide A, luteolin-7-*O*-α-1-rhamnoside and (-)-5¹-methoxy-isolariciresinol-2α-*O*-β-d-xylopranoside have been isolated from the leaves and twigs of *Khaya senegalensis* (Yaun *et al.,* 2013). Quercetin glycosides have been isolated from *Khaya senegalensis* leaves and is the first report of luteolin and iso rhamnetin glycosides in the plant and also khayonolideshave also been isolated (Elsayed *et al.,* 2015). However, information on *Khaya senegalensis* leaves are limited (Abdel-Wareth *et al*., 2014).

2.10.3 Medicinal Uses

Khaya senegalensis (KS) has numerous medicinal applications including antimalarial and antibacterial effects. The stem back extract has been shown to be toxic to *Plasmodium falciparum* (Ademola *et al*., 2004). Moreover, it is well known that the stem bark of *Khaya senegalensis* possesses some anti-sickling (Fall *et al*., 1999), anti hyperglycemic (Kolawole *et al*., 2012), anti microbial (Sale *et al*., 2008), anti fungal (Abdelgaleil *et al*., 2004), anti protozoal

(Ibrahim *et al*., 2013), anti helminthic effects (Ademola *et al*., 2004; Ndjonka *et al*., 2011) and anti cancer (Androulakis *et al*., 2006; Zhang *et al*., 2009). It is known to have free scavenger activities (Bhattacharjee and Bhattacharyya, 2013). Bark in decoction or pulp is taken in most territories as a strong emetic and purgative where treatment is considered to require violent catherisis as in fever, menstrual disorders, venereal diseases, worm infection, stomach upsets, dysentery, poisoning (Burkill, 1985). A cold water infusion may be used externally as a body wash or as a cleansing lotion for dermatitis, sores, ulcers or on small pox pustules to hasten eruption (Burkill, 1985; Mann *et al*., 2003). Black spots on the foot, perhaps verruca are treated in the Gambia with a piece of pounded warm bark (Burkill, 1985). The flowers are sometimes used medicinally for stomach complaints and as an ingredient of anti -syphilitic prescription (Burkill, 1985). The seed is oily and the Fulani in Northern Nigeria use the oil as an ointment for influenza and rheumatism (Burkill, 1985). Leafy twig ends are decocted in Burkina Faso to prepare a wash for skin complaints followed by a dusting of finely powdered bark. Circumcision wounds are treated in like manner. A decoction of leafy tips is used in Ghana to maturate small pox pustules (Burkill, 1985). Salka tribe of North-West Nigeria uses them in stomach medicine (Burkill, 1985).

2.10.4 Veterinary Uses

A cold infusion is given to horses for internal complaints associated with mucal diarrhoea, etc (Burkill, 1985). Together with "Kanwa" (potash) or with "boi-boi" (a sort of salt, south-west Niger), a bark preparation is given to horses as an appetizer. Cattle with liver flukes are given an infusion as bark steeped in bran and water in which corn has been soaked called kasari in Hausa language of Nigeria as a drink. Dried and powdered bark is used as a dressing for ulcers on the backs of camels, horses, etc as also a bark decoction in a lotion (Burkill, 1985). In Nigeria, bark

of the locust bean tree *Butyrospenum parkii* or *Datura metal* (Hauka-ta yaro zakami) fruit, *K. senegalensis* bark together with red peppers are administered in drinking water for poultry to drink *ad libitum* for general prevention of diseases (Gefu *et al*., 2000). The bark of *K. senegalensis* is used to treat emaciation and helminthosis, amoebic dysentery, and diarrhoea in poultry (Gefu *et al*., 2000). The use of mahogany (*K. senegalensis*) seed oil in the control of ticks is also wide spread in Northern Nigeria (Gefu *et al*., 2000).

2.10.5 Domestic Uses

The wood of this species was probably the first African mahogany to be exported from West Africa on a commercial scale in the early part of the Nineteenth century. *K senegalensis* produces good cabin wood (Shukla and Misra, 1994). It is commonly used for making canoes (Burkill, 1985). The straight boles sawn into fine planks and if seasoned carefully, does not suffer shakes. It is attractively figured and is an excellent wood for high grade carpentry and joinery. It has wide scale use for furniture, gunstocks, ship and boat construction and conversion into ply wood and veneers. Mortars and drums are also made from it. House hold spoons are made from it in Western Sudan (Burkill, 1985). The wood converts into good quality charcoal ash from calcined wood which is used as a preservative dressing on millet seeds in store for sowing the following year. In like manner oil from the seed is used as an insectifuge. Saw dust is urticant (Burkill, 1985). The bark holds a pale greenish yellow to golden yellow gum-kino. On slashing, it is expissated sometimes very freely and over a long period. In the dry season, the agglomeration at the base of trees is collected. Catechuic tannins are also presents at about 10% concentration and used for dyeing and tanning. Hunters in Ghana use this dye named kuntunkuri (Asante) to dye their clothing to a suitable brown colour (Burkill, 1985). Young twigs are used in Sudan as toothbrushes. Peeled stem or the roots are used by Hausas in Northern Nigeria as

chewing sticks (Burkill, 1985). The leaves are given to cattle and camels as fodder. Branches of trees are lopped in Kaduna (Nigeria). The foliage is said to be not particularly palatable but to have high protein content 18.7% or 21.7% (Burkill, 1985).

2.11.0 Phytochemistry

The most important application of phytochemical investigation methods is in the field of pharmacognosy which focuses on natural products and the components thereof that show biological activity and are therefore used in therapy (Hajnos *et al*., 2008). The history of phytotherapy is almost as long as the history of civilization (Hajnos *et al*., 2008). The Assyrian-Egyptian, Chinese and Greek records of great antiquity make reference to the nature and use of herbs and herbal drugs. Knowledge of medicinal plants spread in West Europe and then in the whole of Western world to a large extent through the monasteries and their schools of medicine. In the 16^{th} century, early botanists published herbals usually illustrated with the wood cut pictures. In modern science, phytotherapy appeared in the 19^{th} century when the first biologically active compounds (basically alkaloids) were isolated from the plant material (example Morphine, Strychinine, Narcotine, Caffeine etc). The golden age of phytotherapy lasted until 1935, when the 1^{st} sulphonamides and then antibiotics were synthesized and used in therapy (Hajnos *et al*., 2008). The age of chemotherapy began; however, it is a widely recognized fact that numerous synthetic drugs exert along with a positive therapeutic effect, also harmful and often irreversible side effects. Due to this, in recent years, a return to phytotherapy has been observed (Hajnos *et al*., 2008). This return has further been spurred by an appeal by the World Health Organization to screen plants for presence of biologically active compounds contained therein. It is firmly believed that a great yet still not fully revealed therapeutic potentials exists in plants because so far only a few percent out of 250,000 plant species have been investigated with regard to their

usefulness in medicine (Hajnos *et al*., 2008). Nowadays medicines of natural origin are appreciated for their high effectiveness and low toxicity and they are the widely used as commercial products (Hajnos *et al*., 2008). The market value of herbal preparation selling in the United States alone is estimated at several dozen million dollars per year (Hajnos *et al*., 2008).

2.11.1 Chromatography

Chromatography involves the distribution of a compound between two phases - a moving mobile phase that is passed over an immobile stationary phase. Separation is based on the characteristic way in which compounds distribute themselves between the two phases.

For compound X, this can be described in terms of its distribution coefficient

$$KD = \frac{[X]\ \text{Stationary phase}}{[X]\ \text{Mobile phase}}$$

This is characteristic for a molecule which is independent of the amount of solute, so that as one phase carrying the solute passes over the stationary phase, the solutes are in constant dynamic equilibrium between the two phases (Cannell, 1998). For any given compound, the position of this equilibrium is determined by the strength interaction of the compound with the stationary phase and the competition for the stationary phase between the compound and the mobile phase. The stationary phase may be a solid or a liquid and the fluid mobile phase may be liquid or gas to give liquid chromatography and gas chromatography respectively (Cannell, 1998). Liquid chromatography is widely used for preparative isolation of natural products and it comes in different forms (Cannell, 1998).

2.11.1.1 Classification of liquid chromatography

Classification of liquid chromatography can be done in the following manner

1. Classification on the basis of the physical arrangement of the systems. Chromatography can be carried out in the form of a column (Column chromatography) or on a flat surface (planner chromatography). The latter includes various forms of Thin layer Chromatography and Paper chromatography. Countercurrent chromatography involves two liquid phases. Most chromatography is carried out in the form of a column of stationary phase with a moving liquid mobile phase and comprises everything from capillary column to high pressure liquid chromatography (HPLC) and large scale gravity-fed columns (Ikan, 2008; Cannell, 1998).
2. Classification according to mode of separation. It is perhaps more useful to divide chromatographic forms according to the mode of separation on which each is based. (Cannell, 1998).

2.11.1.2 Procedures of Thin Layer Chromatography

Among the chemical methods of plant separation, chromatographic analysis plays a very important role and has been introduced to all the modern pharmacopoeias. Because of numerous advantages of the chromatographic methods (such as their specificity and a possibility to use them for qualitative and quantitative analysis) they comprise an integral part of the medical plant analysis (Hajnos *et al*., 2008; Ikan, 2008).

The following chromatographic methods are most frequently applied in phyto chemical analysis:

a. One – and two dimensional paper chromatography

b. One and two dimensional thin layer chromatography (TLC: also called planner chromatography)

c. High performance column liquid chromatography (HPLC)

d. Gas chromatography (GC) and

e. Counter current chromatography (CCC) (Hajnos *et al*., 2008).

Drug discovery efforts from plants has been evolving continuously in due response to a number of recent technological advances such as the development of chromatographic methods that allow reproducible and fast purification steps for diverse compound classes.

2. 11.1.3 Thin Layer Chromatography

Planar liquid chromatography involves separation of mixtures on thin layers of adsorbents that are usually coated on glass, plastic or aluminum sheet (Cannell, 1998). The most common form of planar liquid chromatography is thin layer chromatography (TLC) and this technique is the easiest, cheapest and most widely used method of the isolation for natural products and is the oldest form of chromatography (Cannell, 1998).

2.11.1.4 Basic Principles of TLC

Separation by TLC is effected by application of the mixture or extract as a spot or thin line onto an adsorbent that has been applied to a backing plate. Analytical TLC plates (0.1-0.2mm) are commercially available; commonest analytical silica gel plate is the 20x20cm plastic or aluminum backed Kiesel gel 60 F_{254} plate which has a 0.2mm thickness of silica sorbent (Cannell, 1998). The plate is then placed into a tank with sufficient suitable solvent to just wet the lower edge of the plate/sorbent but not enough to wet the part of the plate where the spots were applied (origin). The solvent front then migrates up the plate through the sorbent by capillary action, a process known as development (Cannell, 1998).

An important factor in quantifying migration of a compound on a particular sorbent and solvent system is the R_f value: which is defined as

$$R_f = \frac{compound\ \ distance\ \ from\ \ origin\ \ (mid\ \ point}{solvent\ \ distance\ \ from\ \ origin}$$

R_f values are always ratios and are never greater than 1 and vary depending on the sorbent and/or solvent system (Cannell, 1998). These values are sometimes quoted as h R_f i.e. relative to solvent front = 100, h R_f = Rf x 100. Polar compounds have a higher affinity for the sorbent (stationary phase), stick to the sorbent and move slowly up the plate as solvent (mobile phase migrates). The compounds will have relative small R_f values. Non polar compounds have less affinity for the stationary phase, will move comparatively quickly up the plate and therefore have relatively larger R_f values (Cannell, 1998). As a consequence of development, compounds of a mixture will separate according to their relative polarities. Polarity is related to the type and number of functional groups present on a molecule capable of hydrogen bonding (Cannell, 1998).

2.11.1.5 Application of TLC

Traditionally, analytical TLC has found application in the detection and monitoring of compounds through a separation process. In the case of known natural products or other compounds, qualitative and quantitative information can be gathered concerning the presence or absence of a metabolite or breakdown product (Ikan, 2008; Cannell, 1998).

Natural products can be tracked by running analytical TLC of fractions from other separation processes such as column chromatography or HPLC.

2.11.2 Fractionation

All separation processes involve the division of a mixture into a number of discrete fractions. These fractions may be obvious, physically discrete divisions such as two phases of liquid-liquid extraction or they may be the contiguous eluate from a chromatography column that is artificially divided by the extractor into fractions (Cannell, 1998).

The type of fractionation depends on the individual sample and the aims of the separation. Typically, a column is run and the eluate divided into manageable number of even sized fractions followed by analysis of the fraction to determine which contains the desired compounds (Cannell, 1998).

2.11.3 Retention

Solutes only move down the column when they are in the mobile phase moving at the same speed as the mobile phase. The rate of migration of a solute therefore is inversely proportional to its distribution coefficient (Cannell, 1998). The degree of retention of a solute can be described by three retention parameters- retention time (t_R), retention volume (V_R) and capacity factor (K^1). The retention time is the time that a solute spends in the stationary phase plus the time it spends in the mobile phase.

2.11.4 Types of Stationary Phases.

Silicon gel is perhaps the most widely used stationary phase for the separation of natural products (Cannell, 1998). It is a three dimensional polymer of tetrahedral units of silicon oxide (chemically represented by $SiO_2.H_2O$). Being a porous material, it offers very large surface areas (Cannell, 1998). The chemical nature of the surface of silica gel consists of exposed silanol

groups. These hydroxyl groups are the active centres and potentially can form strong hydrogen bonds with compounds being chromatographed (Cannell, 1998). Polar compounds containing carboxylic acids, amines or amides are strongly adsorbed on silica gel. Non polar compounds such as terpenes or other compound lacking polar functional groups contain few hydrogen bonding sites and are thus poorly to non retained on silica gel (Cannell, 1998).

Other types of stationary phases include:

1. Polyacrylamide which are synthetic polymers formed as beads for chromatography,
2. Carbohydrates: Polysaccharides can be cross linked to produce three dimensional networks which can be formed into beads.
3. Polystyrene which are often used for ion exchange resins. However, in the absence of ionizing groups, the polymer can form gel resins useful for reverse phase chromatography.
4. Alumina which is a porous polymer of aluminum oxide. It is no longer commonly used due to its ability to catalyze a variety of reactions (Cannell, 1998).

2.11.5 Spray Detection

This relies on a colour reaction between the compound on the TLC plate and a spray reagent (stain) introduced onto the plate as a fine mist from a spray canister. Most sprays reagents are universal reagents and will react with many classes of natural product. Five percent (5%) Ferric chloride in 0.5N HCl (0.5N Hydrochloric acid) is used for the detection of phenolics (Cannell, 1998).

2.12.0 *In vivo* method for evaluating plant with Anti protozoal activity

In vivo is usually conducted within a living organism. *In vivo* research is more suited to observe an overall effect than in *in vitro* research which is better suited to deduce mechanisms of actions.

2.13.0 *In vitro* method for evaluating Plant with Anti protozoal activity

In vitro assay is an evaluation technique for the assessment of the pharmacological action of either newly synthesized conventional drug or that of crude extract from a plant intended for use on either newly synthesized conventional drug or that of crude extract from a plant intended for use on either animal or man usually carried outside a biological system (ie. Petri dishes or other medium) on the target organisms in order to validate their efficacy or otherwise harmful effects before introduction into a biological system (Simon *et al*., 2011).

Chapter Three

3.0 Materials and Methods

3.1 Fractionation of the crude methanol extracts and their *in vitro* activities on *Eimeria tenella*

3.1.1 The study Area:

The experiments were performed in the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine and the Department of Pharmacognosy and Drug Formulation, Faculty of Pharmaceutical Sciences, Ahmadu Bello University (latitude 11° 10¹ N and longitude 07° 38¹ E), Kaduna state located in the northern Guinea Savannah zone of Nigeria (Akpa *et al.*,2002). The climate is relatively dry with a mean annual rainfall ranging from 700 to 1400 millimeters (mm) and occurring between the months of April and September. The dry season begins about the middle of the month of October with the col weather that ends in February. This is followed by a relatively hot, dry weather from March/April. The minimum and maximum daily temperatures are 14C and 24C respectively during the cold season and 19C and 36C respectively during the hot, dry season. The relative humidity varies between 19% and 35% in the dry season and between 63% and 80% in the rainy season (Alphonsus *et al.*, 2012).

3.1.2 Plant collection and Identification:

Fresh leaves of *Khaya senegalensis* (KS) *Gueira senegalensis* (GS) and *Tamarindus indica* (TI) were sourced from the environs of Ahmadu Bello University, Zaria. They were collected between the months of March and April due to the high bioactive content during these months (Ademola and Eloff, 2010) and they were dried under shade. Ten kilogramme (10kg) of the dried

leaves of each plant with voucher numbers 900181, 900141 and 900265 respectively (Otu *et al*., 2009) were ground to powder using mortar and pestle.

3.1.3 Plant Extraction

3.1.3.1 Defatting of the leaves of *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica:*

Two (2kg) kg each of the powdered leaves of KS, GS and TI were defatted with 6 litres of petroleum ether in a Soxhlet apparatus (Quick fit Corning Ltd; Stafford, England) at 70°C (Youn *et al*., 2003). The powdered leaves were then air- dried at room temperature.

3.1.3.2 Crude plant extraction by maceration method

The powdered leaves were extracted by maceration method for 72 hours using 70% methanol (Eloff, 1998). The crude extract was evaporated to dryness on a water bath at a temperature of 90^0C for 6 hours and subsequently referred to as crude extract.

3.1.4 Fractionation of crude methanol extracts:

The method of Brain and Turner (1975) was adopted for the partitioning process of the dried crude methanol extracts. Thirty (30 g) each of the dried crude methanol extracts of KS, GS and TI was suspended in 300 ml of water and then partitioned in succession using petroleum ether, chloroform and n-butanol to obtain the aqueous methanol portions. The suspended crude methanol extracts were each partitioned with three portions of 300 ml petroleum ether respectively using separate funnels. The petroleum ether portions were each carefully separated into 3 labeled clean 1000 ml beakers. Subsequently, the aqueous methanol portions were partitioned with three portions (300 ml) each of chloroform; followed by three portions of n-butanol. The portions were then referred to as petroleum ether, chloroform, n-butanol and aqueous portions respectively (Figure 3.1).

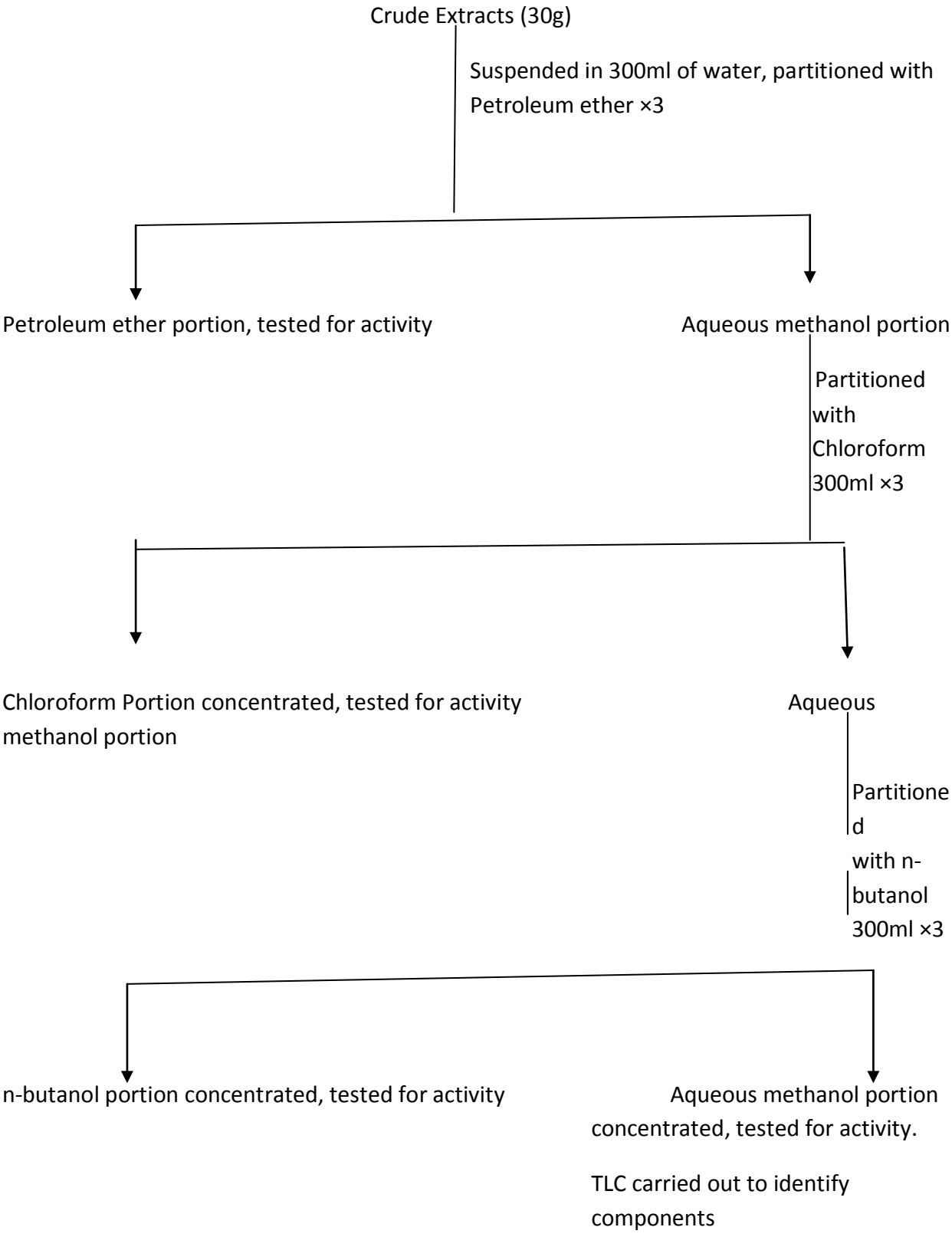


Figure 3.1: Schematic diagram illustrating the partitioning of the various fractions from the crude methanol extract of *Khaya senegalensis*, *Guiera senegalensis* and *Tamarindus indica*

3.1.5 Phytochemical analysis

The phytochemical components of the separated portions of the extracts were then eludated by Thin Layer Chromatography (TLC) according to the method of Agrawal and Paridhavi, (2007) and Brain and Turner, (1975). Silica gel coated thin layer chromatography plates were marked with a pencil drawn at 1.5cm (centimeter) to identify the origin. Using a micro haematocrit capillary tube(non heparinised), the fractions to be tested (which were as follow; Pet-ether, chloroform, n-butanol and the crude extract of *Khaya senegalensis* which was dissolved in methanol) were drawn into the tube and separate spots made on the 1cm mark dot on the plate. The spots were allowed to air dry for 15 minutes and were then placed in a TLC jar containing the test solvent system which in this case was n-butanol: acetic acid: water at the ratio of 6:1:2 (Plate 3.1). The plates placed in the TLC jar were allowed to saturate for 15minutes before the set up was allowed to run while being monitored (Plate 3.1). The plates were removed and a pencil was used to mark the end point. Pictures were taken before and after the detecting agents were sprayed on the plate .The following detecting agents were used to spray the plates; Vanillin in 10% hydrogen sulphate, 10% hydrogen sulphate and Ferric chloride.

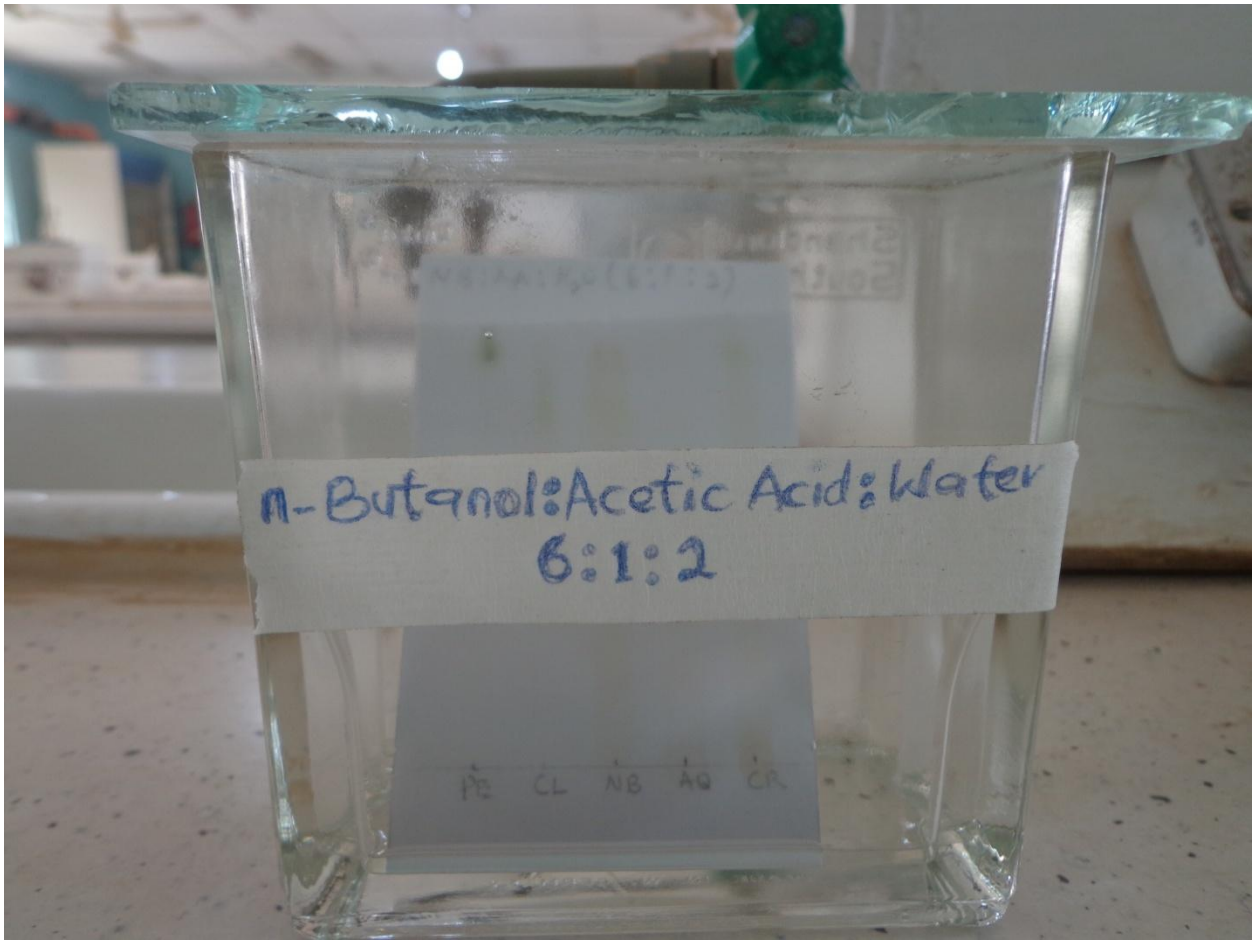


Plate 3.1: Thin Layer Chromatography Tank containing TLC plate eluted in the solvent system of n-butanol, acetic acid and water (6:1:2)

3.1.6 *In vitro* anti coccidial effects of the extracts:

The solvents were evaporated and the residue portions were tested for *in vitro* anticoccidial activities. The *in vitro* anti coccidial efficacy trials was conducted by observing the effect of the plant extracts on the sporulation time of the *Eimeria tenella* oocysts. Fresh faecal samples were collected from experimentally infected birds and the oocysts load was determined using the McMaster counting machine. Using the method described by Nwosu *et al*. (2011) the extracts were diluted (100 mg/ml, 250 mg/ml, 400 mg/ml, 550 mg/ml, 700 mg/ml and 850 mg/ml of aqueous and n-butanol fractions respectively) in distilled water and placed in separate labeled petri dishes. One hundred oocysts per ml calculated by dilutions of the total oocyst load was added to each petri dish and left at ambient temperature on the laboratory table, monitored every 6 hourly for 72 hours to observe sporulation and sporulation time of the oocysts. The rate of sporulation of the oocysts were determined and recorded. The findings were used to determine percentage inhibition of oocysts sporulation. A parallel experiment using laboratory sporulation medium (2.5% Potassium dichromate) and Amprolium (1.2g/L) were used for comparison as negative control and positive control respectively.

3.2 Determination of maximum tolerated dose and chronic toxicity of *Khaya senegalensis* extracts in broiler birds

3.2.1 Maximum tolerated dose experiment:

The method described by Loomis (1978) was used to determine the maximum tolerated dose (MTD). Sixty (60) day-old chicks were sourced from NAPRI, Shika, Zaria, brooded under standard management practice for 4 weeks and divided into 6 groups namely A, B, C, D, E and F. Graded doses of the n-butanol fraction of the crude methanol plant extracts of *Khaya*

senegalensis 11 mg/kg, 33 mg/kg, 99 mg/kg, 297 mg/kg and 891 mg/kg were administered orally to the birds in the respective groups. Group F were given 0.2 mls of distilled water as control. All the extracts were administered within the same period. The chickens were monitored for 14 days for adverse reactions such as changes in skin, feathers, ocular membrane, salivation, lacrimation, drowsiness, gait tremors, convulsion and death suggestive of toxicity within 72 hours. The chickens that survived were monitored for another two weeks after which the test was terminated. Two chickens per group were randomly selected, euthanized and post mortem examinations conducted. Organs examined for lesions included the lungs, heart, liver, spleen, kidneys, intestine and caeca.

3.2.2 Chronic toxicity studies:

Two groups of 7 birds each (1 and 2) of 4 weeks of age broilers were used for the chronic toxicity study. Group 1 chickens were orally dosed with 500mg/kg of the n-butanol fraction of the crude methanol plant extracts of *Khaya senegalensis* daily for two weeks. Group 2 served as the control group and each was given distilled water at 0.2ml for the same period of time. The chickens were monitored for signs such as salivation, lacrimation, drowsiness, gait tremors, convulsion and death suggestive of toxicity for the period of the study. At the end of 12 weeks, 2 birds from each of the groups were randomly selected, euthanized and post mortem examination conducted. Gross lesions were noted and tissue samples of the following organs (lungs, heart, liver, spleen, kidneys, intestine and caeca) were taken for histopathology.

3.3.0 Evaluation of the modulatory effect of the n-butanol fractions of the crude methanol extracts of *K senegalensis* on *E. tenella* in broilers

3.3.1 Experimental birds:

One hundred and twenty day old chicks were obtained from the National Animal Production Research Institute (NAPRI), Shika, Zaria. The birds were reared under standard management practice at the Faculty of Veterinary Medicine A.B.U. Zaria for a period of 8 weeks. The birds were brooded for 3 weeks and then randomly divided into six groups namely A, B, C, D, E and F of 20 chicks each. Birds in each group were further subdivided into two replicates with each having 10 chicks. All the birds were fed with Broiler starter rations for 3 weeks and Broiler finisher rations from 4 weeks to 8 weeks. The chicks were provided with standard anticoccidial-free feed. The feed and water were provided *ad libitum* during the study period. All the birds were tagged to maintain their identity.

3.3.2 Administration / Treatment of the experimentally infected birds:

Three dose levels which are 11mg/kg, 33mg/kg and 99mg/kg were selected from the maximum dose experiment.

Group A: The birds were given *Khaya senegalensis* at 11mg/kg.

Group B: The birds were given *Khaya senegalensis* 33mg/kg

Group C: The birds were given *Khaya senegalensis* 99mg/kg.

Group D: The birds were treated with Amprolium (1.2g/L).

Group E: The birds were infected with sporulated *E tenella* oocysts and not treated (0.2mls water).

Group F: The birds were not infected with sporulated *E tenella* oocysts and were given 0.2mls of distilled water.

3.3.3 Inoculum:

Infective sporulated *E tenella* oocysts were obtained locally from infected broiler birds processed sporulated and kept in the refrigerator (+4°C). Oocysts from the intestinal scrapings were sporulated using 2.5% potassium dichromate. The sporulated oocysts were filtered using a sieve, centrifuged (750g) for 10 minutes to remove the potassium dichromate and diluted with distilled water (Reck and McQuistion, 1994). The sporulated oocysts were kept in the refrigerator (+4°C).

3.3.4 Infection of birds with the sporulated oocysts:

The chicks in groups A,B,C.D and E were infected orally at 4 weeks of age with 1.0×10^5 sporulated oocysts / ml / bird using insulin syringe (Reck and McQuistion, 1994). Group F was given 0.2ml of distilled water.

3.3.5 Anticoccidial:

Amprolium (Amprolium-20S VMD nvlsa Hoge Mauw 900-2370, Arendonk, Belgium) was used as the standard drug to compare the anticoccidial efficacy of the extract.

3.3.6 Post infection treatment with the n-butanol plant extract was as follows:

Groups A, B and C were given calculated dosages of the n-butanol methanol plant leaves extracts twice daily for 7 days, while Group D was given Amprolium. However, Group E was

not treated with the plant extract (positive control) while group F was given 0.2mls of distilled water only.

3.3.7 Laboratory Monitoring of Infection:

The experimental infection of the birds with the sporulated oocysts and subsequent treatment with the plant extract were monitored as follows:

3.3.7.1 Collection and Examination of faecal samples:

Faecal samples were collected from the birds in each group daily using clean polythene bags as from 4 to 8 weeks of age and examined for the presence of *Eimeria tenella* oocysts. The oocysts output was determined and expressed as per gram of faeces using McMaster counting chamber (Sloss *et al*., 1994).

3.3.7.2 Collection of blood samples for haematology:

Blood samples were collected weekly from each group one week post infection using the wing veins till the end of the experiment and analyzed for Packed Cell Volume, haemoglobin concentration, total / differential leukocyte count and total protein.

3.3.7.3 Body weight:

The body weight gain with respect to age was determined on weekly basis from weeks 4 to 8.

3.3.7.4 Post mortem Examinations

i Gross Pathology:

Two birds from each of the 6 groups were selected at random 7 days post treatment and sacrificed humanely using cardiac air pumped. Birds were also sacrificed humanely from the positive and negative control groups for post mortem examination. The carcasses were examined for pathological lesions and samples such as the intestines and caeca were taken for histopathology. They were stored in 10% formalin. Appropriate photographs of the gross lesions were also taken.

ii: Histopathology:

Slides for histopathologic examination were prepared at the Histotechnique laboratory of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. Tissues were processed routinely and embedded in paraffin wax. Paraffin sections of about 5µm-6µm were prepared. Deparaffinization in xylene and dehydration in graded series of ethanol was followed by staining with Haematoxylin and Eosin stain. Appropriate photographs of the histopathologic lesions were also taken.

3.4.0 Assessment of response to various experiments included:

(i) Oocysts (count, sporulation time, infectivity), (ii) Haematology (PCV, Total Protein, Haemoglobin concentration, Differential and Total leukocyte count), (iii) clinical signs, (iv) weight, (v) feed intake, (vi) mortality and morbidity, (vii) Gross pathology and histopathology.

3.5.0 Statistical Analysis:

Data obtained from the experiment were analyzed with Analysis of Variance (ANOVA) using SAS statistical package of General linear model and P values of < 0.05 were considered to be statistically significant.

Chapter Four

4.0 Results

4.1 Extraction of plant material and partitioning of crude extracts

The extraction of 2kg of the leaves of *Khaya senegalensis* (KS), *Guiera senegalensis* (GS) and *Tamarindus indica* (TI) with 70% methanol as solvent gave a yield of 436.42g (21%), 496.41g (24.82%) and 394.20g (19.71%) respectively. The colour of the extract was dark brown for both KS and GS while TI had a reddish brown colour (Table 4.1).

The extractive yield output of the partitioned portions of the crude methanol extracts of *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica* when Petroleum ether, chloroform and n-butanol were used as partitioning solvents for the partitioning of 30g of crude methanolic gave the following; 0.01g (0.03%), 0.25g (0.83%), 6.73g (22.43%) for KS, 0.03g (0.1%), 0.12g (0.4%), 6.83g (22.77%) for GS and 0g (O%), 0.33g (1.1%), 4.5g (15%) for TI. The aqueous yield were as follows; 13.47g (44.9%), 8.65g (28.83%), and 8.58g (28.6%) respectively for KS, GS and TI. The petroleum ether portion was greenish in colour for KS and GS and very faint colour of green for TI was. The chloroform, n-butanol and aqueous methanol portions were brown, reddish brown and brown respectively for KS and GS and reddish brown for TI (Table 4.2).

Table 4.1: Crude extractive yield of the leaves of *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica* used in the study

Plant	*Khaya senegalensis*	*Guiera senegalensis*	*Tamarindus indica*
Plant part collected	Leaves	Leaves	Leaves
Quantity of Plant material collected	10kg	10kg	10kg
Quantity of Plant sample extracted	2kg	2kg	2kg
Output of crude extract/Percentage	436.42g	496.41g	394.20g
Percentage yield after Extraction	(21%)	(24.82%)	(19.71%)
Colour of Extract	Dark brown	Dark brown	Reddish brown

Table 4.2: Fractional yield output of *K. senegalensis, G senegalensis* and *Tamarindus indica*

Plant	Fractionated Portions			
	Petroleum ether/ Colour	**Chloroform/ Colour**	**n-butanol/ Colour**	**Aqueous Fraction/ Colour**
Khaya senegalensis	0.01g (0.03%) Greenish	0.25g (0.83%) Brown	6.73g (22.43%) Brown	*13.47 (44.9%) Brown
Guiera senegalensis	0.03 (0.1%) Greenish	0.12 (0.4%) Brown	*6.83 (22.77%) Brown	8.65 (28.83%) Brown
Tamarindus indica	0 (O %) Faint Green	0.33 (1.1%) Brown	4.5 (15%) Reddish Brown	8.58 (28.6%) Reddish Brown

4.2 Phytochemical analysis

The thin layer chromatography plate took 90minutes to run in the TLC tank. The retention factor was determined. The petroleum ether spot had a retention factor of 0.47cm, chloroform $R_{f;}$ 0.61, n-butanol R_f; 0.70 and the crude extract of *K senegalensis* R_f; 0.84 (Table 4.3). The TLC plate was sprayed with ferric chloride as the detecting agent which gave greenish coloured spots which signified the presence of phenolic compounds. The greenish colourations were mainly seen in the n-butanol and crude extract fractions of the plant (Plate 4.1). All the other detecting agents used did not yield any positive colouration.

Table 4.3: Retention factors for the compounds present in the n- butanol fractions of *Khaya senegalensis* extracts.

Fractions	Distance travelled by Spot (S) in cm	Distance travelled by Solvent Front (S_of) in cm	Retention factor (R_f) in cm
Petroleum ether	3.5	7.6	0.46
Chloroform	4.6	7.6	0.61
N-butanol	5.4	7.6	0.70
Aqueous	6.4	7.6	0.84
Crude extract	6.9	7.6	0.91

Keys: cm- centimeter

$Sof = 7.6cm$

$$Spa = 3.5cm = \frac{3.5}{7.6} = 0.46cm$$

$$Spb = 4.6cm = \frac{4.6}{7.6} = 0.61cm$$

$$Spc = 5.4cm = \frac{5.4}{7.6} = 0.71cm$$



Plate 4.1: TLC plate after spraying the various fractions eluted in the solvent system (6:1:2) with Ferric chloride. Note the greenish spots indicationg the presence of phenols and flavonoids.

4.3 *In vitro* Study

The n-butanol suspension for *Khaya senegalensis* investigated showed that no sporulation occurred in any of the concentrations (100 mg/ml, 250 mg/ml, 400 mg/ml, 550 mg/ml, 700 mg/ml and 850 mg/ml) while the aqueous fraction had sporulated oocyst present in all the concentrations except the 100 mg/ml group (Plates 4.2 and 4.3). The n-butanol suspension for *Guiera senegalensis* (400 mg/ml, 550 mg/ml, and 700 mg/ml) and *Tamarindus indica* (250 mg/ml and 400 mg/ml) as well as the aqueous fraction of *Guiera senegalensis* did not inhibit the sporulations of the *E tenella* oocysts (Plates 4.4 to 4.6 and Table 4.3). However, the aqueous fraction of *Tamarindus indica* had inhibited sporulation. The sporulation medium (2.5% Potassium dichromate) did enhance sporulation of the oocysts (Plate 4.7).

The n-butanol fraction of the *Khaya senegalensis* had the most anticoccidial activity among the fractions tested. The extract inhibited sporulation by 100% while *Guiera senegalensis* had the least anti coccidial activity with 49% sporulation inhibition. Amongst aqueous fractions, *Tamarindus indica* showed the most anticoccidial activity with a sporulation inhibition of 100% while *Khaya senegalensis* and *Guiera senegalensis* had the least. The n-butanol fraction of *K senegalensis* and the aqueous fraction of *Tamarindus indica* extracts had similar effects to those obtained using amprolium which was considered as the standard. Amprolium completely inhibited the sporulation of the oocysts in this study (Table 4.4).



Plate 4.2: Sporulating *Eimeria tenella* oocyst (arrow) in aqueous fraction of *Khaya senegalensis* extract (700 mg/ml) at × 40 magnification.



Plate 4.3: Unsporulated *Eimeria tenella* oocyst (arrow) in n- butanol fraction of *Khaya senegalensis* extract (850 mg/ml) at × 40 magnification



Plate 4.4: Sporulating *Eimeria tenella* oocyst in aqueous fraction of *Guiera senegalensis* leaf extract (250 mg/ml) at × 40 magnification

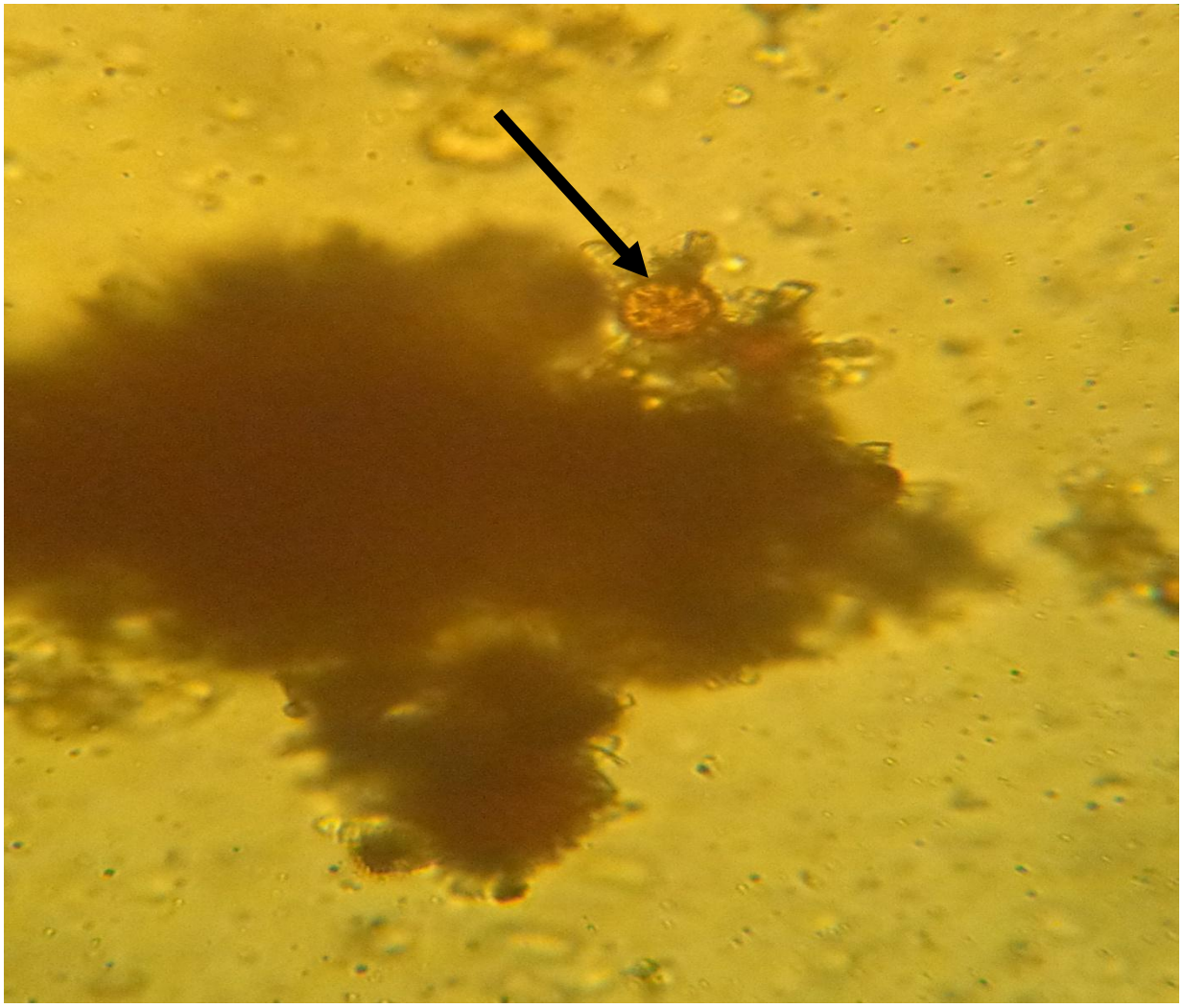


Plate 4.5: Sporulating *Eimeria tenella* oocyst in n-butanol fraction of *Guiera senegalensis* leaf extract (850 mg/ml) at × 40 magnification



Plate 4.6: Sporulated *Eimeria tenella* oocyst (arrow) in aqueous fraction of *Tamarindus indica* leaf extract (400 mg/ml) at × 40 magnification



Plate 4.7: Sporulated *Eimeria tenella* oocyst (arrow) in 2.5% Potassium dichromate sporulation medium at × 40 magnification

Table 4.3: The effects of n-butanol and aqueous leaf extracts of *Khaya senegalensis, Guiera senegalensis and Tamarindus indica* extracts on sporulation of the *Eimeria tenella* oocysts

Drug /Extract	Concentration(mg/ml)	Percentage Sporulation (%)	
		n-butanol Fraction	Aqueous Fraction
Khaya senegalensis	100	0	0
	250	0	17
	400	0	17
	550	0	17
	700	0	17
	850	0	17
Guiera senegalensis	100	0	17
	250	0	17
	400	17	17
	550	17	17
	700	17	17
	850	0	0
Tamarindus indica	100	0	0
	250	17	0
	400	17	0
	550	0	0
	700	0	0
	850	0	0

Amprolium	1.2mg/ml	0	-
Potassium dichromate	2.5% solution	0	-

Table 4.4: Percentage Sporulation Inhibition of n-butanol and aqueous leaf extracts of *Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica*

Drug/Extract	Fractions	Percentage (%)		Total
		Sporulation	Sporulation Inhibition	
Khaya senegalensis	n-butanol	0	100	100
	Aqueous	85	15	100
Guiera senegalensis	n-butanol	51	49	100
	Aqueous	85	15	100
Tamarindus indica	n-butanol	34	66	100
	Aqueous	0	100	100
Amprolium	-	0	100	100
2.5% Pottassium dichromate	-	100	0	100

4.4 The Maximum tolerated dose by the birds on the different concentrations of the Extracts

There were no visible clinical sign seen except during the administration of the extract to the birds being given the 891mg/kg dosage which showed signs of respiratory difficulty but stabilized after 30minutes.

4.4.1 Gross Lesions

Several macroscopic lesions were identified in the posted infected birds. These varied in the intensities and location on the concentration /doses of the extract administered. At 11 mg/kg of the n-butanol fraction, the lesions were mainly those of enlarged proventriculus and echymotic haemorrhages of the serosal surface of the duodenum. Enlargement of the liver characterized the gross lesions associated with the administration of 33 mg/kg and 99 mg/kg doses of the extract. Higher doses of 297 mg/kg and 891 mg/kg were associated with enlarged liver, congested spleen and enlarged heart (Plates 4.8 and 4.9). The organs of the control birds presented normal gross picture (Plate 4.10).

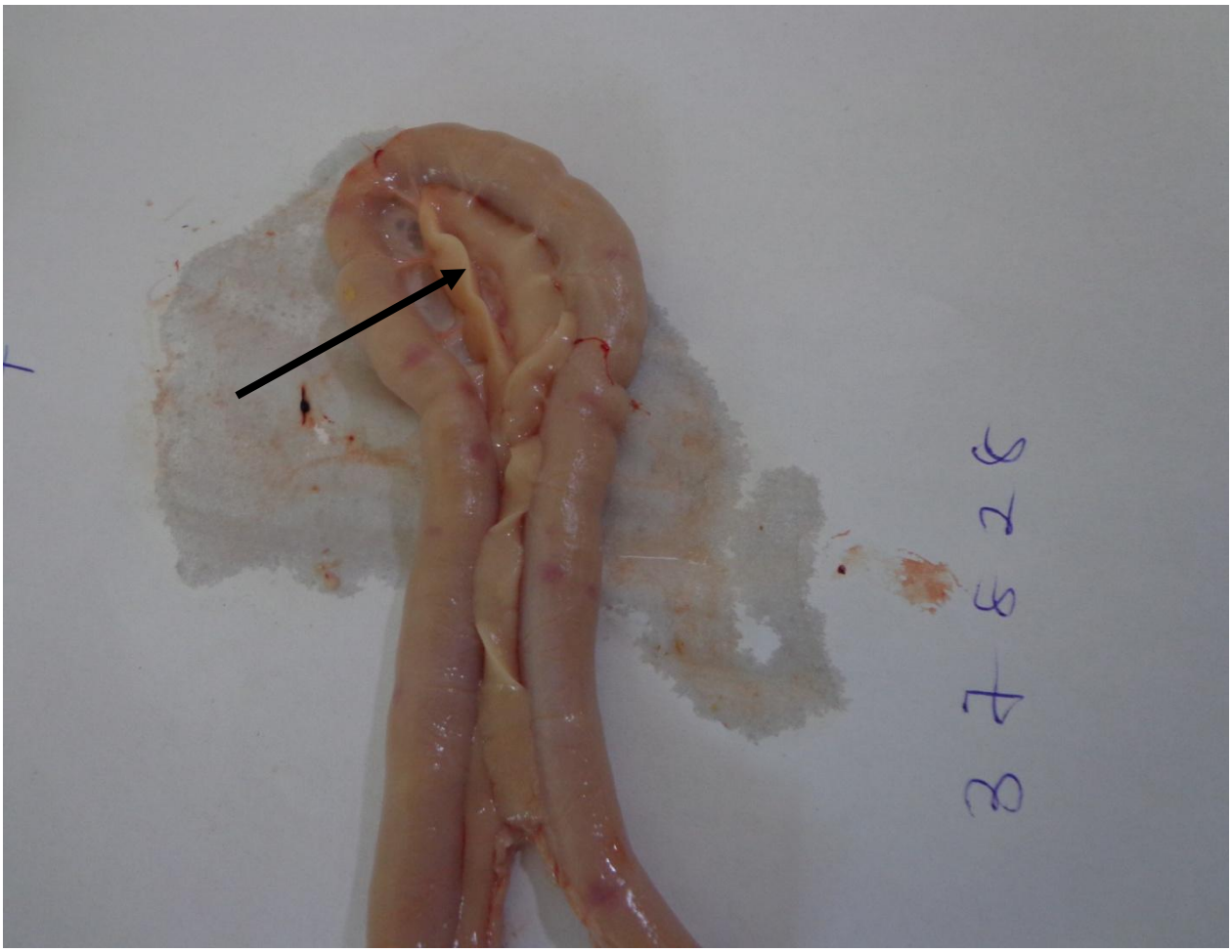


Plate 4.8: Small intestine of broiler chicken dosed with the n-butanol fraction of the extract (11 mg/kg) *Khaya senegalensis* showing enlargement of the intestinal wall (arrow)

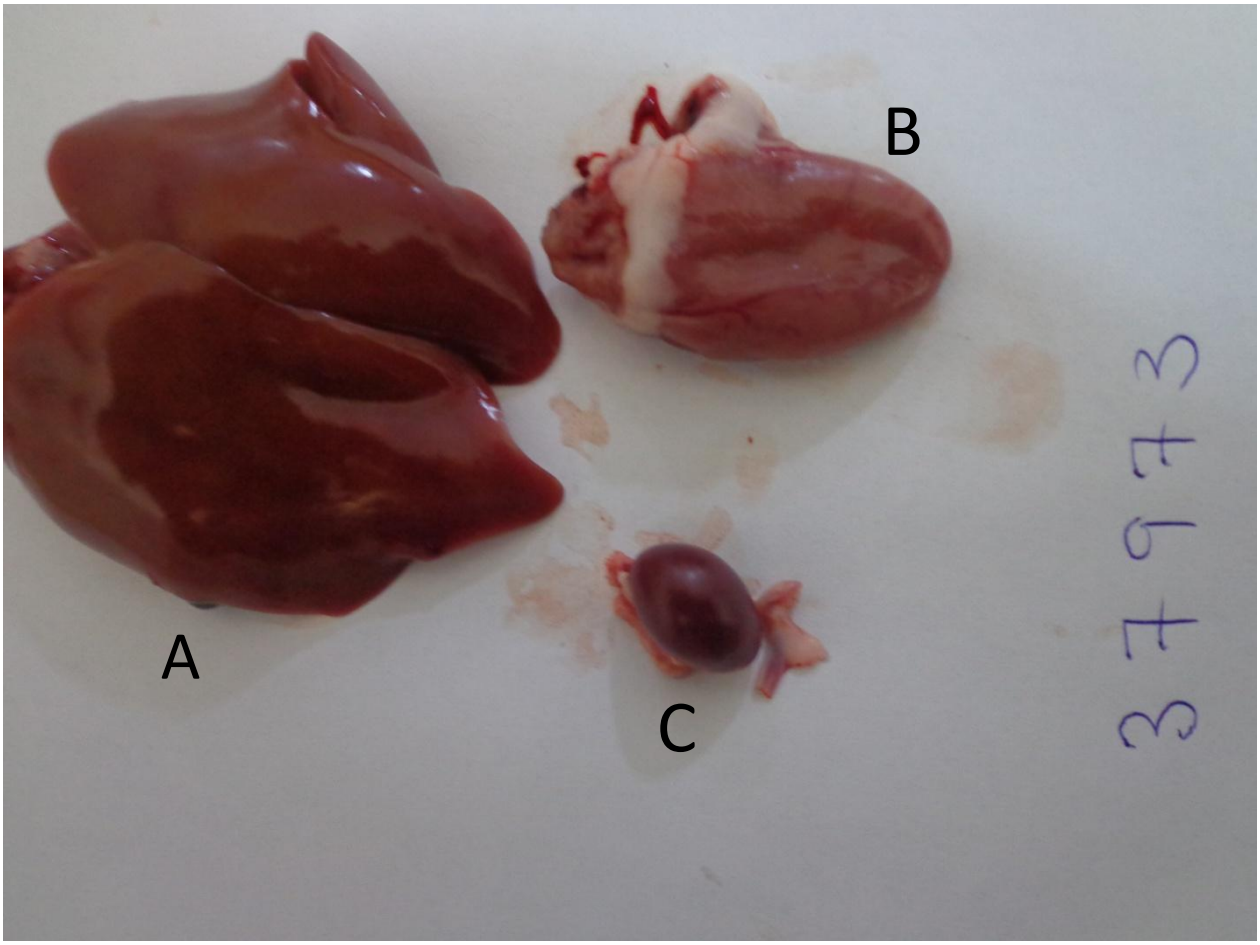


Plate 4.9: The liver (A), heart (B) and spleen (C) showing enlargements of the organs of birds dosed with 297 mg/kg of *Khaya senegalensis*

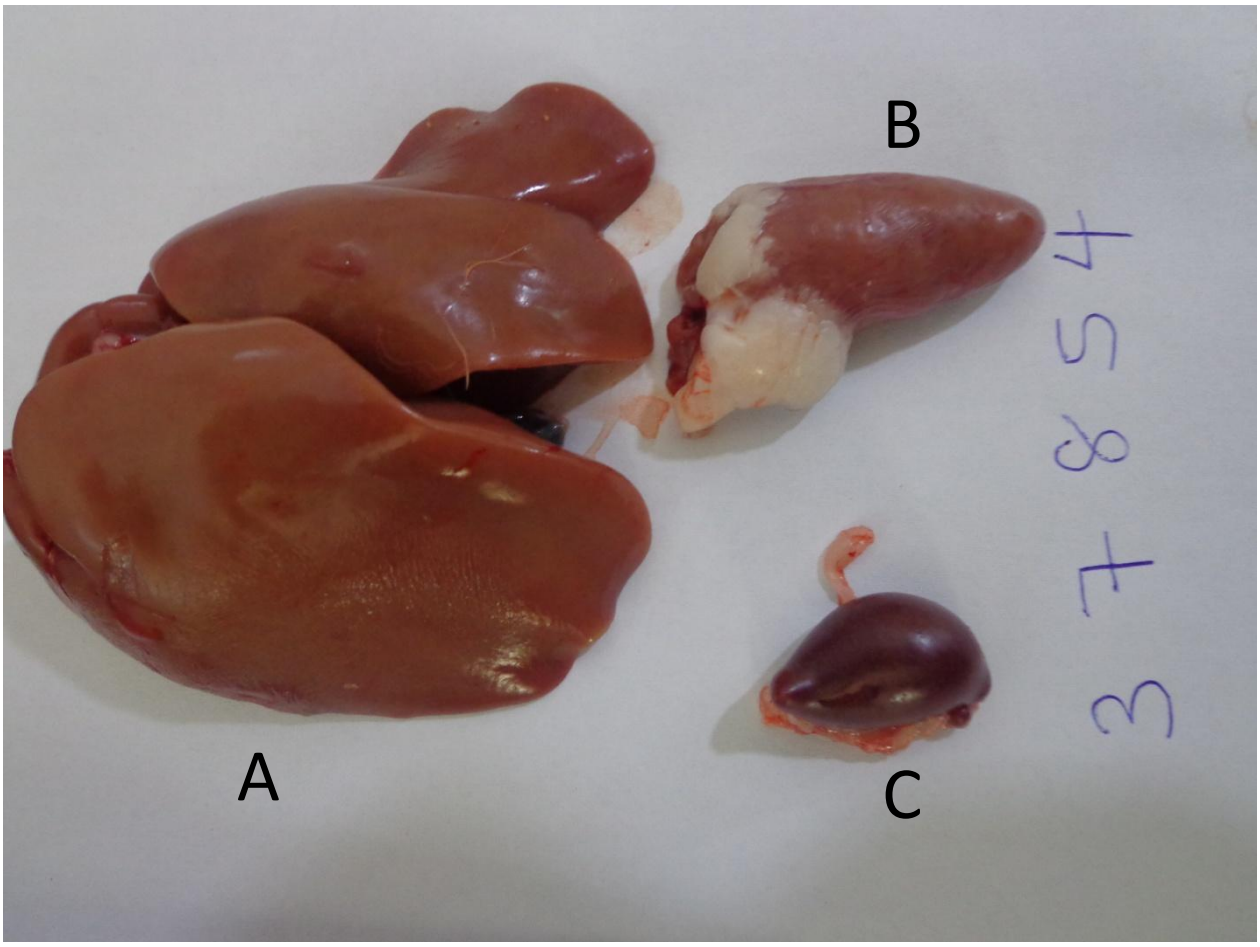


Plate 4.10: Normal gross features of the liver (A), heart (B) and spleen (C) of the control broiler chickens given water (0.2ml/ bird)

4.4.2 Haematological values of broiler birds dosed given different doses of the n-butanol extract of *Khaya senegalensisi* in the maximum tolerated dose study

There was statistically significant difference ($P < 0.05$) when the mean PCV values for the different treatment groups (11, 33, 99,297,891mg/kg and control) were compared with the mean haemoglobin values. Also when the mean lymphocytic values were compared with the mean neutrophil values there was statistically significant difference ($P < 0.05$). Within the treatment groups, there was no statistically significant difference ($P>0.05$) when they were compared within each other though the PCV of the treated groups were higher than the control (28.14±3.96) with 33mg/kg treated group having the highest (29.15±3.80 vs28.14±3.96). Similarly, the Hb also had slightly higher values than the control with the 33 mg/kg group having the highest (9.69±1.24 vs9.32±1.32). The total protein (TP) was slightly different with the overall values being lower than the control, though the 297 mg/kg treated group had a higher value than the control (3.65±1.11 vs 3.60±0.59). The overall lymphocytic values were higher than the control though the 33 mg/kg group had the highest value while the neutrophil had low values in the 11mg/kg, 33 mg/kg and the 297 mg/kg treated groups while the 99 mg/kg and 891 mg/kg groups had slightly higher values than the control (Table 4.5).

Table 4.5: The effect of the extracts of *Khaya senegalensis* on the haematological values of broilers treated with different doses of the extracts in the maximum tolerated dose study

Variable	Haematological Results (Mean ± SE) P<0.05					
	11 mg/kg	**33 mg/kg**	**99 mg/kg**	**297 mg/kg**	**891 mg/kg**	**Control (water 0.2 ml/bird)**
PCV (%)	$_{a}$28.43±2.72	$_{a}$29.15±3.80	$_{a}$28.88±4.28	$_{a}$28.91±4.51	$_{a}$ 29.02±5.65	$_{a}$ 28.14±3.96
Hb (g/dl)	$_{a}$9.42±0.91	$_{b}$9.69±1.24	$_{b}$9.61±1.43	$_{b}$9.57±1.52	$_{b}$9.63±1.89	$_{b}$9.32±1.32
TP (g/dl)	3.42±0.55	3.47±0.62	3.41±0.62	3.65±1.11	3.38±0.55	3.60±0.59
Lymphocyte (%)	$_{c}$89.73±5.08	$_{c}$91.00±3.46	$_{c}$89.14±4.56	$_{c}$90.96±3.69	$_{c}$89.83±3.78	$_{c}$89.56±3.78
Neutrophil (%)	$_{d}$9.32±5.08	$_{d}$8.24±3.57	$_{d}$9.90±4.24	$_{d}$8.53±3.39	$_{d}$9.70±4.09	$_{d}$9.63±3.93

Keys

SE- Standard Error

P<0.05- Statistically significant

PCV- Packed Cell Volume

Hb- Heamoglobin

TP- Total Protein

a-Means with similar letters are not statistically significant

b- Means with different letters are statistically significant

4.4.3 Weight

The baseline weight of the birds is as shown in Table 4.6. One week after the administration of the extract, the mean weight of the control treatment group when it was compared with other treatment groups (11mg/kg and 33mg/kg) was statistically significant ($P<0.05$). There was statistical significant differences between the mean weights of birds treated with 11 mg/kg of the n-butanol fractions of crude methanol extract of *Khaya senegalensis* compared with 99 mg/kg, 297 mg/kg, 891 mg/kg treated groups and the control ($P<0.05$) two weeks post treatment. However, there were no significant differences in the mean weights between 11mg/kg and 33mg/kg ($P>0.05$). When the 33mg/kg treatment group was compared with 891mg/kg and 297mg/kg groups, there were significant differences in their means ($P<0.05$). Three weeks post treatment showed significant differences in the comparison of the mean weights between 11mg/kg , 297mg/kg, 891mg/kg and the control ($P<0.05$) and four weeks post treatment, there were significant differences ($P<0.05$) in the mean weights of 11mg/kg , 297mg/kg, 891mg/kg treatment groups and the control (Tables 4.7). Among the treated groups, the highest weight gain was recorded in the 11mg/kg treatment group while the lowest was recorded in the 891mg/kg group (Table 4.8).

Table 4.6: Pre-treatment mean weight (grammes) of the experimental birds

Treatment	Weight (grammes) (Mean ± SE) P<0.05
Extract 11mg/kg	449.44± 17.11
Extract 33mg/kg	338.80±16.23
Extract 99mg/kg	303.33±17.11
Extract 99mg/kg	260.40±16.23
Extract 99mg/kg	204.60±16.23
Control	205.86±19.40

Keys:

SE- Standard Error

P<0.05- Statistically significant

Table 4.7: Mean weights (grammes) of birds one to four weeks post treatment with *Khaya senegalensis*

Treatment	Weight (grammes) (Mean ± SE) P<0.05			
	Weeks Post treatment			
	1	2	3	4
Extract 11mg/kg	$_{a}$643.00±24.92	$_{a}$927.00±38.33	$_{a}$1176.00±56.57	$_{a}$ 1450.00±74.46
Extract 33mg/kg	$_{a}$555.00±24.92	$_{a}$804.00±38.33	$_{a}$1060.00±56.57	$_{a}$ 1300.00±74.46
Extract 99/mg/kg	$_{a}$510.00±24.92	$_{b}$693.00±38.33	$_{a}$1048.00±56.57	$_{a}$ 1195.00±74.46
Extract 297/mg/kg	$_{a}$449.00±24.92	$_{b}$600.00±38.33	$_{b}$872.00±56.57	$_{b}$1110.00±74.46
Extract 891/mg/kg	$_{b}$368.00±24.92	$_{b}$619.00±38.33	$_{b}$835.00±56.57	$_{b}$1067.00±74.46
Control	$_{b}$383.00±24.92	$_{b}$671.00±40.40	$_{b}$901.00±63.25	$_{b}$1031.25±83.25

Keys:

SE- Standard Error

P<0.05- Statistically significant

a-Means with similar letters are not statistically significant

b- Means with different letters are statistically significant

Table 4.8: Mean weight gain (grammes) of the experimental birds treated with *Khaya senegalensis* four weeks post treatment

Variable	Weight (grammes) (Mean ± SE) P<0.05		
	Final Weight	**Initial Weight**	**Weight Gain**
Extract 11mg/kg	1450.00±74.46	449.44± 17.11	$*_a$1000.56±57.35
Extract 33mg/kg	1300.00±74.46	338.80±16.23	$_b$961.20±58.23
Extract 99/mg/kg	1195.00±74.46	303.33±17.11	$_b$891.67±57.35
Extract 297/mg/kg	1110.00±74.46	260.40±16.23	$_b$849.6±58.23
Extract 891/mg/kg	1067.00±74.46	204.60±16.23	$_b$862.4±58.23
Control	1031.25±83.25	205.86±19.40	$_b$825.39±63.85

Keys:

SE- Standard Error

P<0.05- Statistically significant

a-Means with similar letters are not statistically significant

b- Means with different letters are statistically significant

***- Highest weight gain**

4.4.4 Survival Rate

The survival rate of birds treated with the n-butanol fraction of the crude methanol extract of *Khaya senegalensis* was 100% even with the highest concentration of 891mg/kg as no mortality was recorded in the treatment groups (Table 4.9).

Table 4.9: Survival Rates of birds treated with different doses the extract *Khaya senegalensis*

Group	No of Birds	Dose (mg/kg)	Pre treatment mortality	Post treatment mortality	Total mortality	Survived Birds	Percentage (%) Survival
A	10	11 mg/kg	0	0	0	10	100
B	10	33 mg/kg	0	0	0	10	100
C	10	99 mg/kg	0	0	0	10	100
D	10	297 mg/kg	0	0	0	10	100
E	10	891 mg/kg	0	0	0	10	100
F	10	0.2ml (water)	0	2	2	8	80

4.4.5 Histopathological Findings

The histopathological lesions showed congestions of the intertubular spaces in the kidneys of the birds treated with the n-butanol fractions of the crude methanol extracts of *Khaya senegalensis* at the doses of 33 mg/kg and the 297 mg/kg (Plates 4.11 and 4.12). The liver of the birds treated with the dose of 891 mg/kg had congestion of the sinusoidal spaces (Plates 4.13). However, no significant findings were seen in the small intestine of the 99 mg/kg group and that of the control group which given water at the dose of 0.2 ml/ bird.

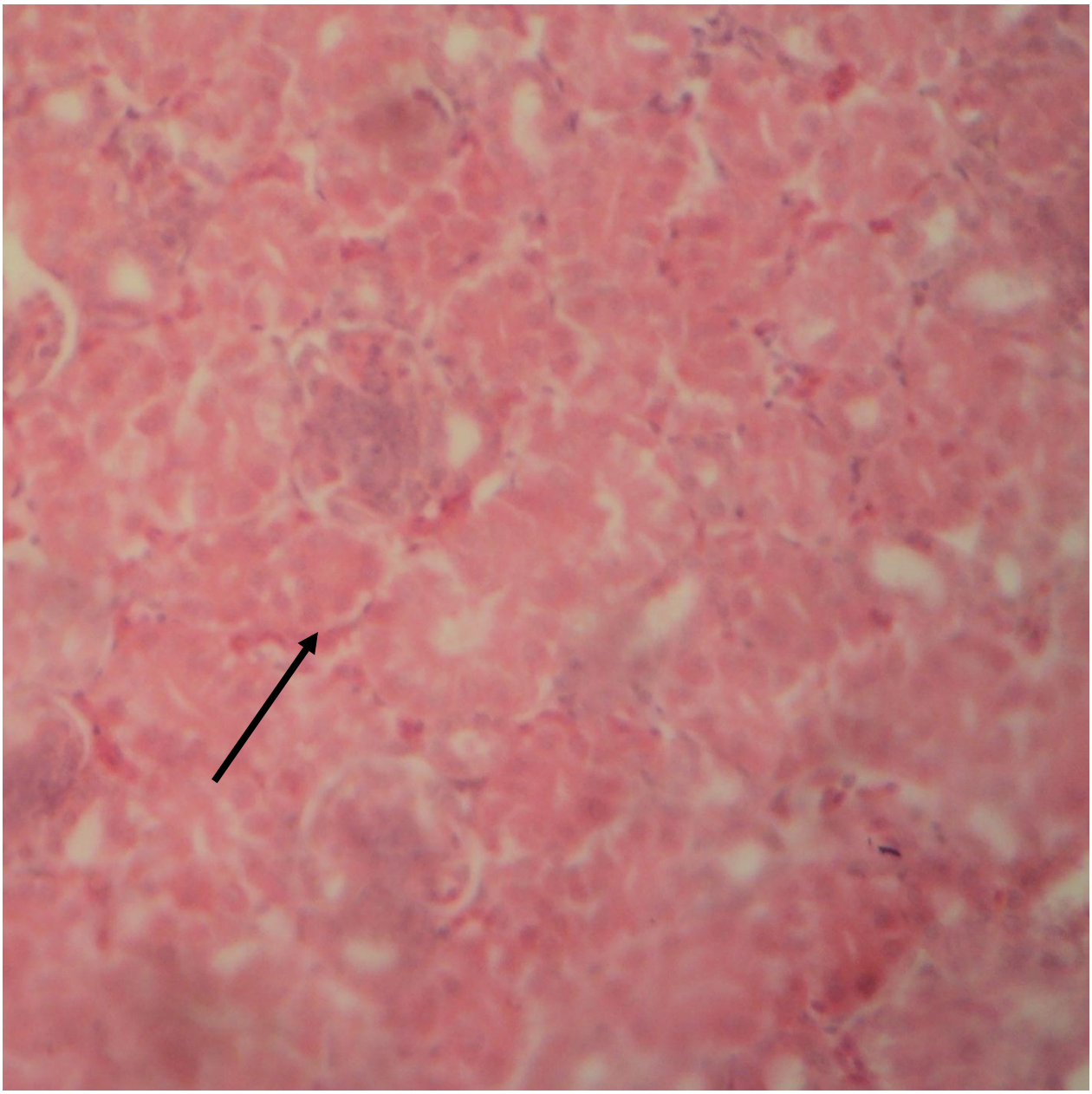


Plate 4.11: Congested intertubular spaces of the kidney of a bird dosed with 33 mg/kg of *Khaya senegalensis* extract (arrow). (H and E) × 40 magnification

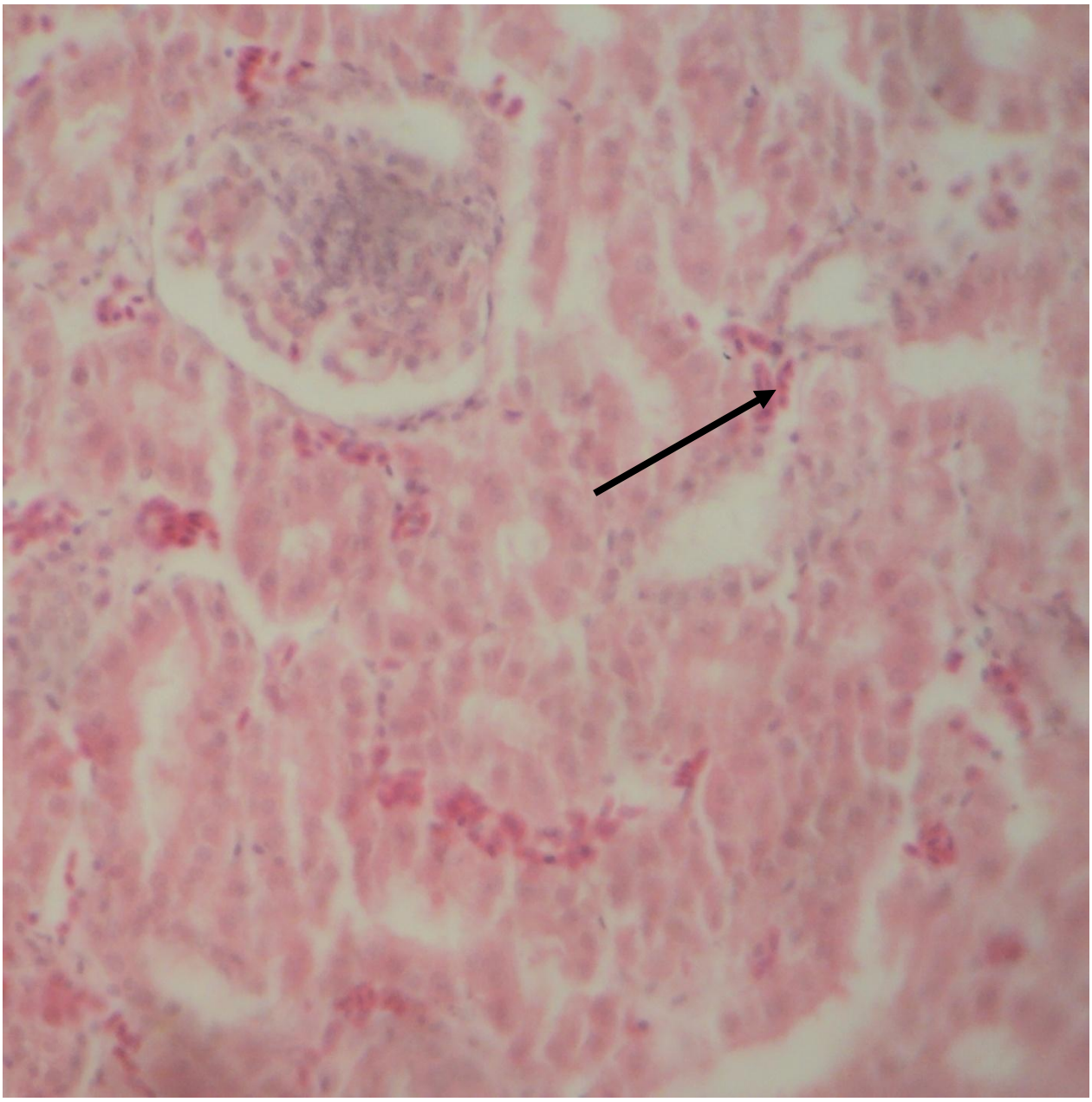


Plate 4.12: Congested intertubular spaces in the kidney of a bird treated with extracts of *Khaya senegalensis* at a dose of 297 mg/kg (arrow). (H and E) × 40 magnification

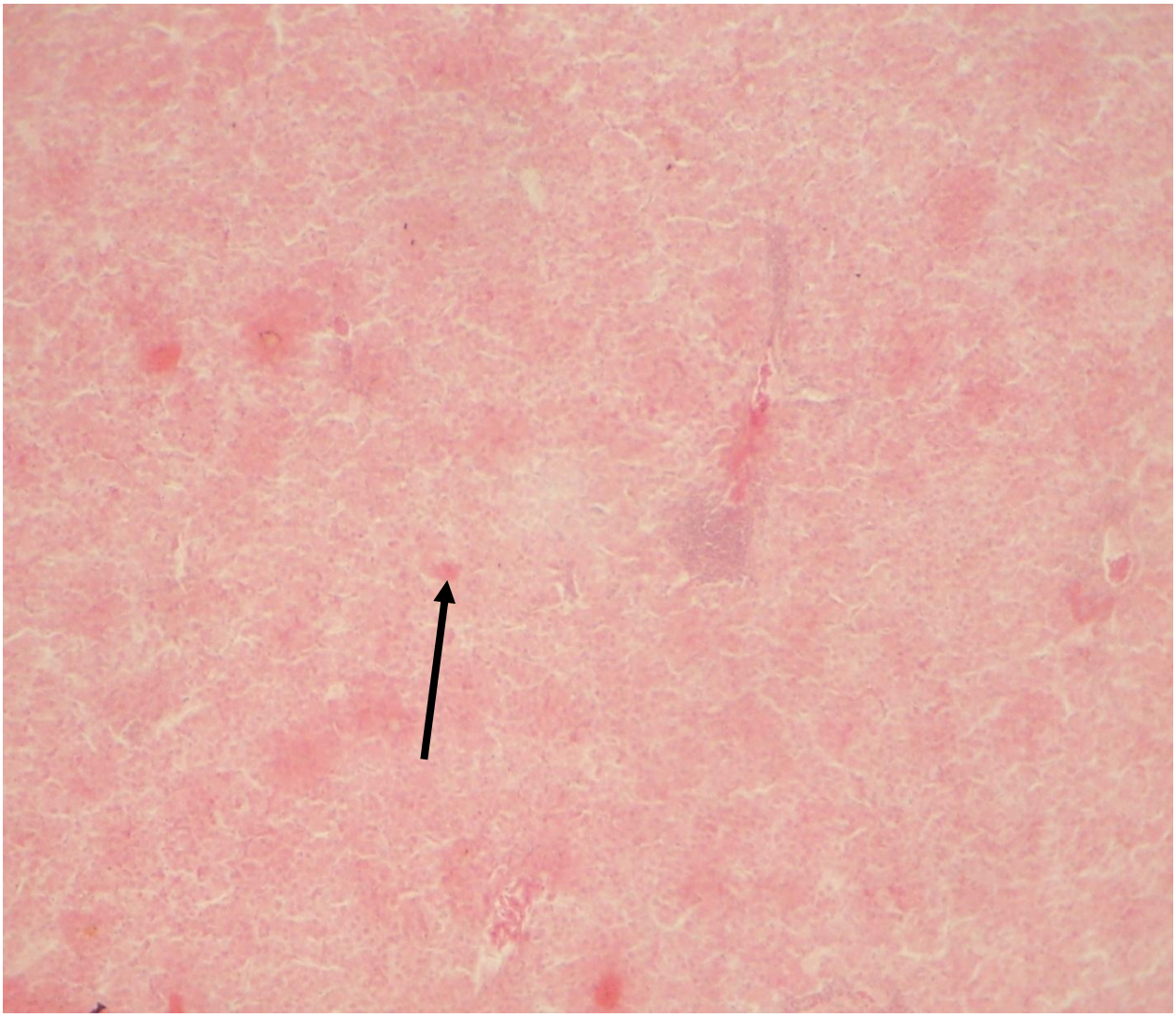


Plate 4.13: Congested sinusoidal spaces in the liver of a bird treated with extracts of *Khaya senegalensis* at the dose of 891 mg/kg (arrow). (H and E) × 40 magnification

4.4.6 Selection of the maximum dose tolerated by the birds

Results obtained from the study revealed that although all the birds were active when they were given the extracts of *Khaya senegalensis*, the 891 mg/kg treated group had respiratory distress but they stabilized after 30 minutes. Also at 4 weeks post administration of the extracts of *Khaya senegalensis*, the 297 mg/kg and 891 mg/kg treated groups had lower weight gains. The 99 mg/kg dose was selected as the maximum tolerated dose. However, three dose levels were selected from the 99 mg/kg (which are as follows; 11 mg/kg, 33 mg/kg and the 99 mg/kg) for the evaluation of the anticoccidial effects of the extracts *in viv*o (Table 4.10).

Table 4.10: Maximum tolerated dose/toxicity of n-butanol fractions of crude methanol leaf extract of *Khaya senegalensis*

Dose (mg/kg)	**11**	**33**	**99**	**297**	**891**	**Control**
Initial no. of birds	10	10	10	10	10	10
Mortality	0	0	0	0	0	2
Observation	a	A	a	ac	abc	a
Inference	-	-	-	+	+	-

Keys: a= active 6hours to 4weeks post treatment, b=showed initial respiratory difficulty for 30minutes after treatment, c= lower weight gain 4 weeks post treatment, - = not toxic, + = mildly toxic

4.5 Administration of the extracts of n-butanol fraction of *Khaya senegalensis* (500 mg/kg) to broilers in the chronic toxicity studies

There were no visible clinical signs observed in broilers treated with 500 mg/kg of the n-butanol fraction of the methanolic extract of *Khaya senegalensis.* They were all active.

4.5.1 Gross Lesion

The gross lesions however, showed hypertrophied, congested kidneys and enlarged, distended and haemorrhagic intestine with thickened intestinal wall in the birds treated with the extract (500mg/kg) at 16 weeks of age (Plates 4.14- 4.16). The duodenum and jejunum had echymotic haemorrhagies in them while the jejunum had mucoid contents. In the control group, all the organs were normal and no lesions were seen in them (Plate 4.17).



Plate 4.14: The *in situ* appearance of the kidneys of a bird treated with n-butanol fraction of the crude methanol extracts of *Khaya senegalensis* (500 mg/kg) for 14 days showing hypertrophied kidneys (arrow).



Plate 4.15: The liver of a bird treated with n-butanol fraction of the crude methanol extracts of *Khaya senegalensis* (500 mg/kg) for 14 days showing enlarged liver (arrow).

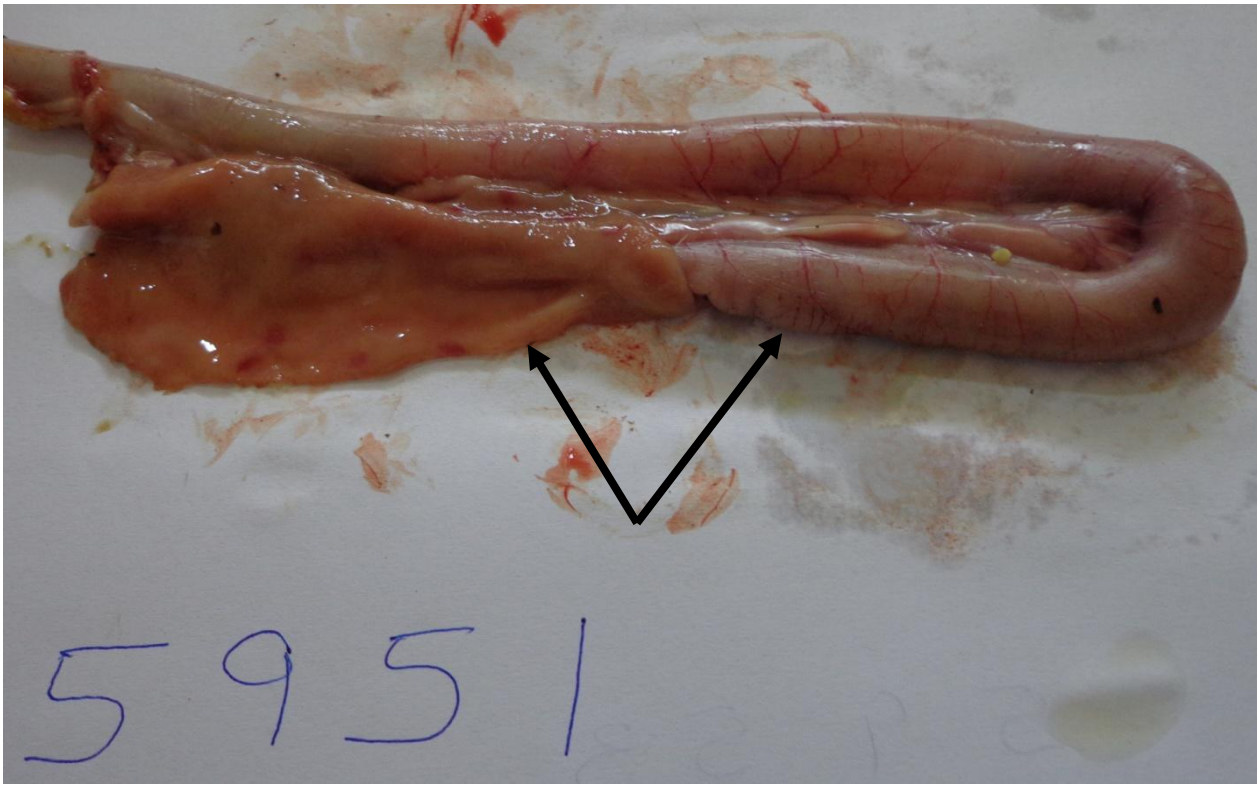


Plate 4.16: The small intestine of a bird treated with n-butanol fraction of the crude methanol extracts of *Khaya senegalensis* (500 mg/kg) for 14 days showing thickened intestinal wall with enlarged and distended intestine (arrows).



Plate 4.17: The small intestine of a bird treated water (0.2 ml/bird) for 14 days showing normal intestinal architecture (arrow).

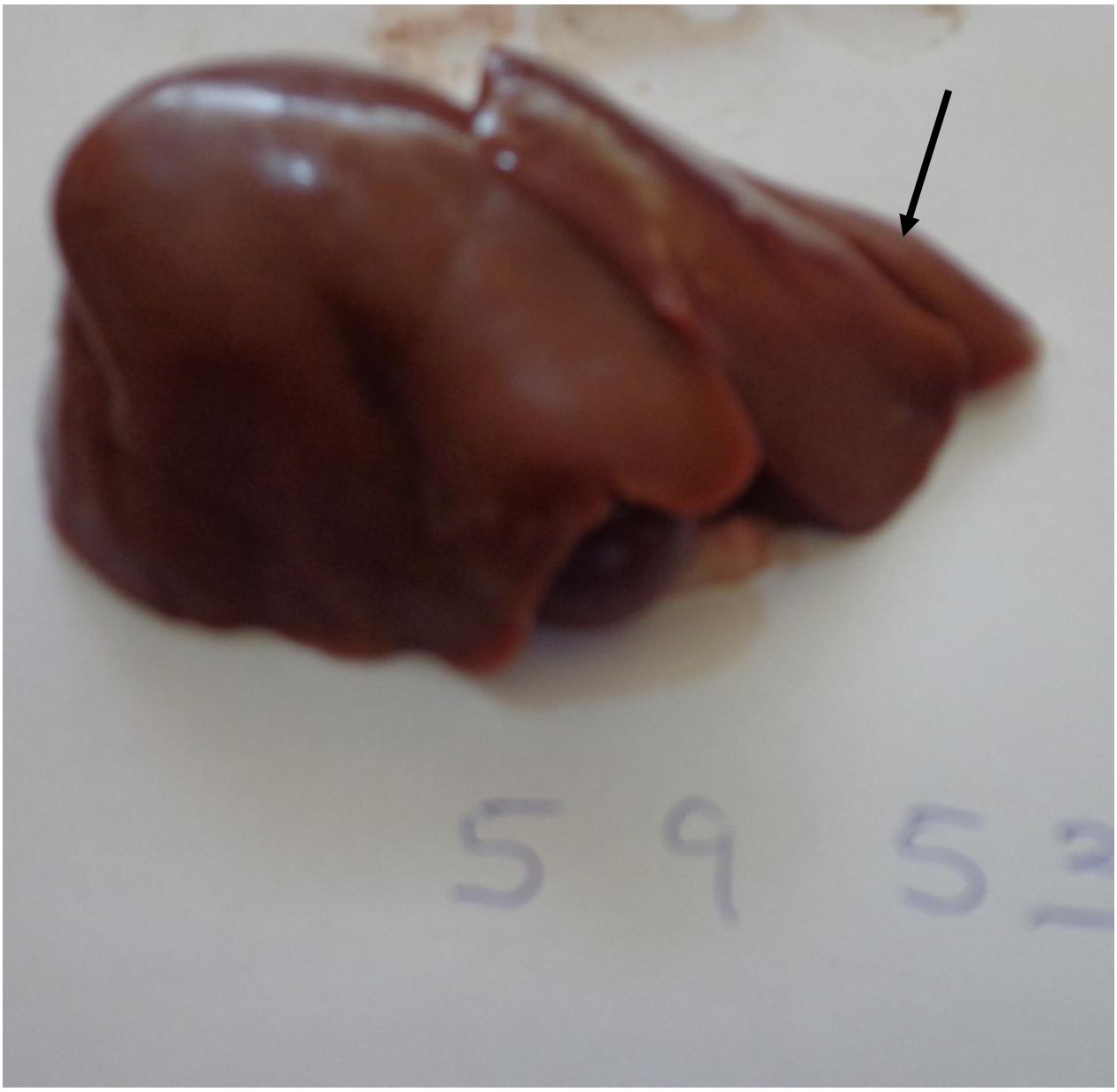


Plate 4.18: The liver of a bird treated water (0.2 ml/bird) for 14 days showing normal architecture (arrow).

4.5.2 Effect of chronic administration of the n-butanol fraction of the crude methanol extracts of *Khaya senegalensis* on haematological parameters of broiler chickens

The PCV of birds treated with the extract (500 mg/kg) was relatively higher but not statistically significant ($P>0.05$) when compared with those in the untreated control group (water 0.2ml) though both groups peaked at week 10. Also the Hb of the medicated group (500 mg/kg) was slightly higher than the unmedicated group and was statistically significant ($P<0.05$) peaking at the tenth week (Figures I and II). The total protein (TP) was slightly higher than the unmedicated group during the first week of medication. However, the TP dropped on the third week thereafter it began to rise, peaking at the tenth week (Figure III). The neutrophils of the medicated group were higher than the unmedicated group during the weeks of medication peaking at the seventh week. The lymphocyte of the medicated groups remained lower than that of the unmedicated group (Figure IV).

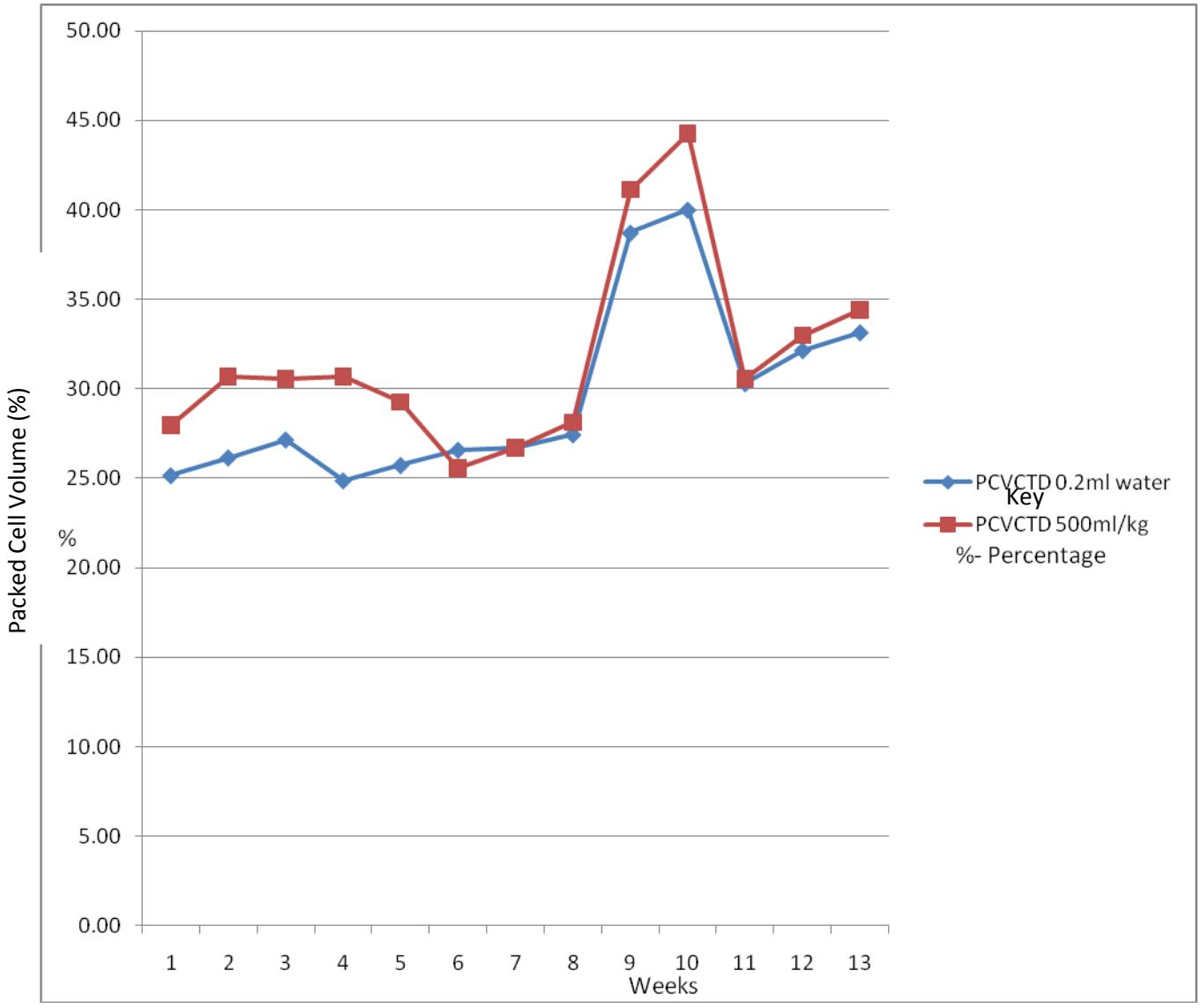


Figure 4.1: Packed Cell volume of broiler chickens treated with a dose of 500 mg/kg of *Khaya senegalensis* for 14 days

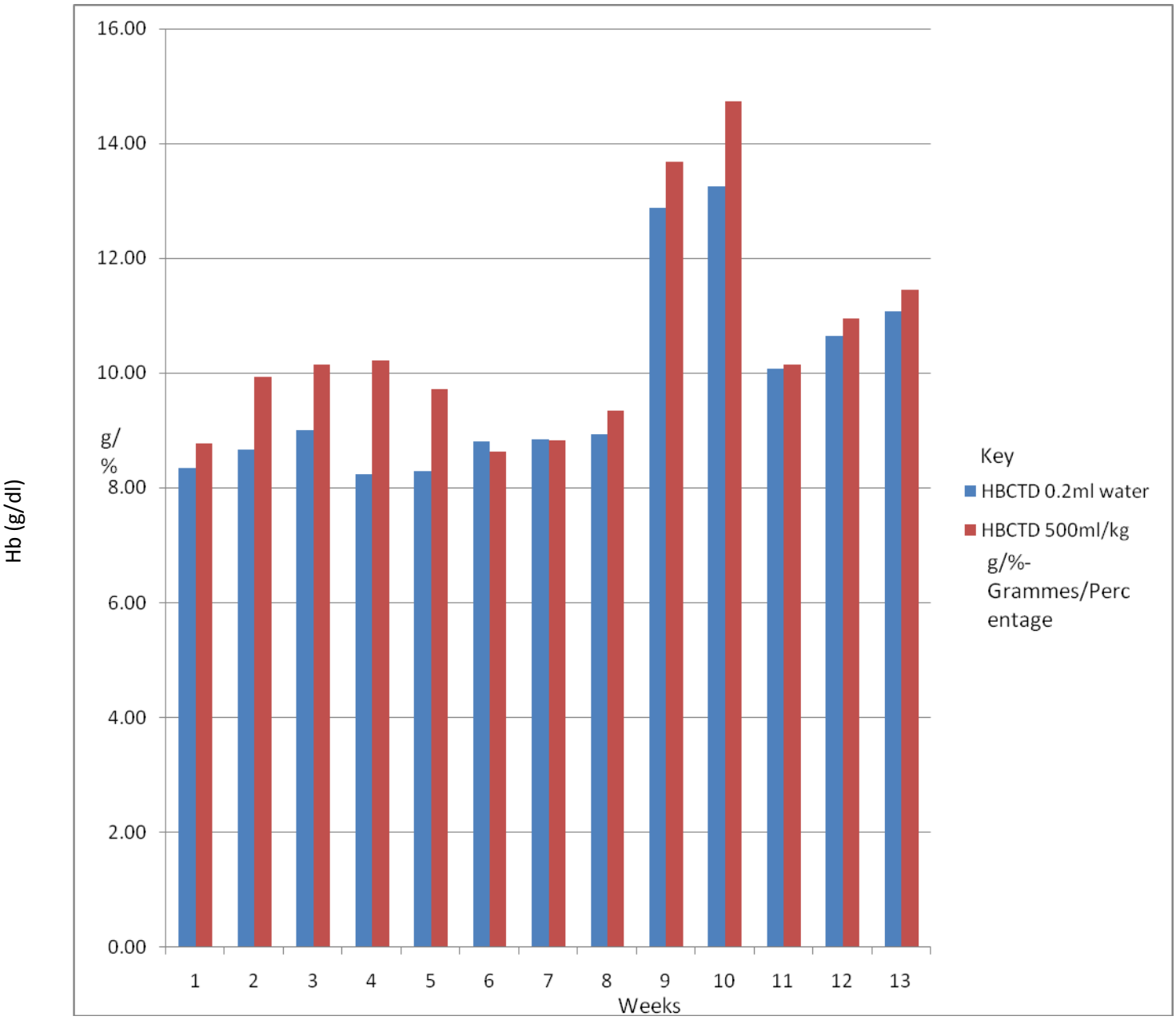


Figure 4.2: Haemoglobin concentration of birds treated with 500 mg/kg of *Khaya senegalensis* for 14 days

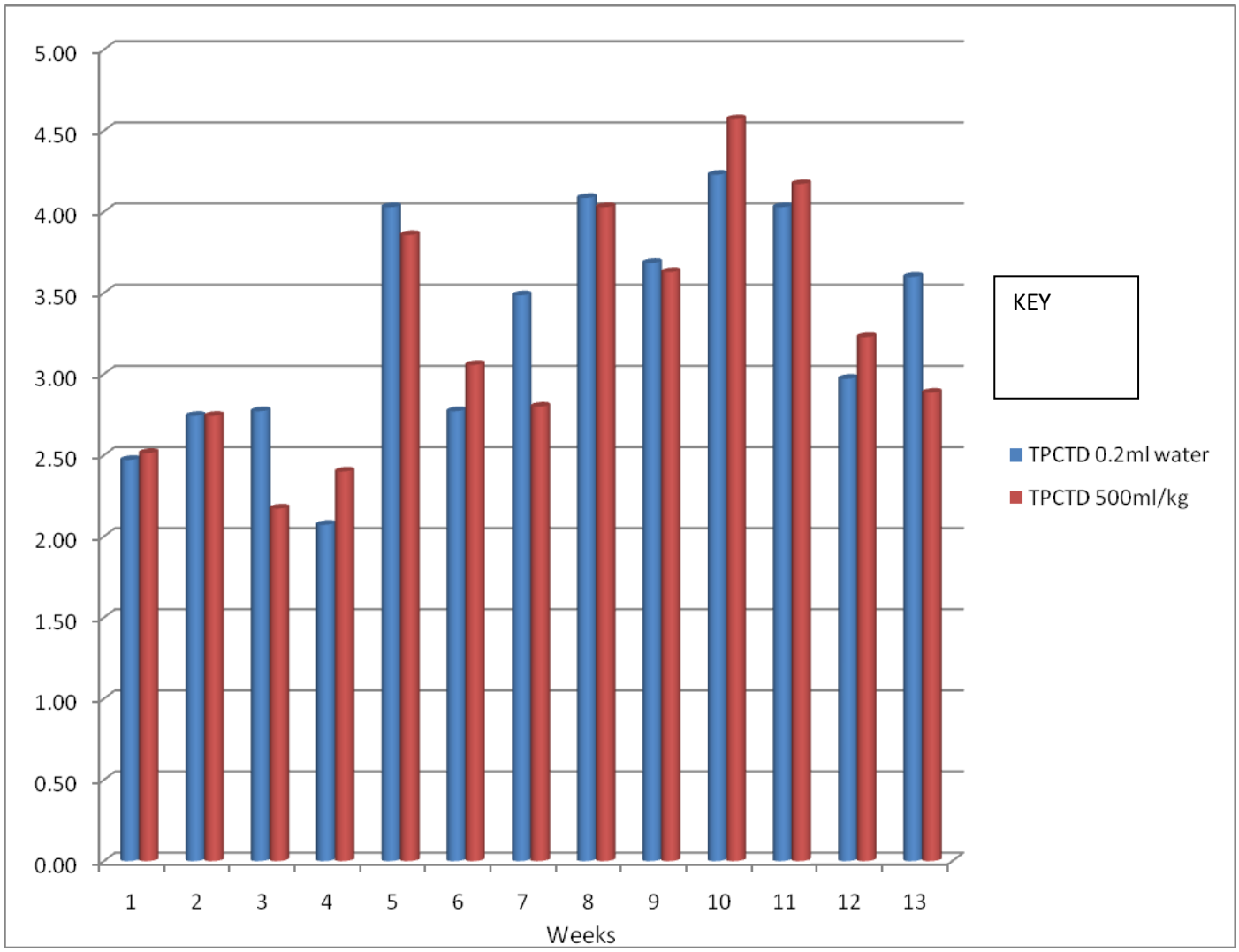


Figure 4.3: Evaluation of the total protein of birds treated with 500mg/kg for a 12 weeks study period

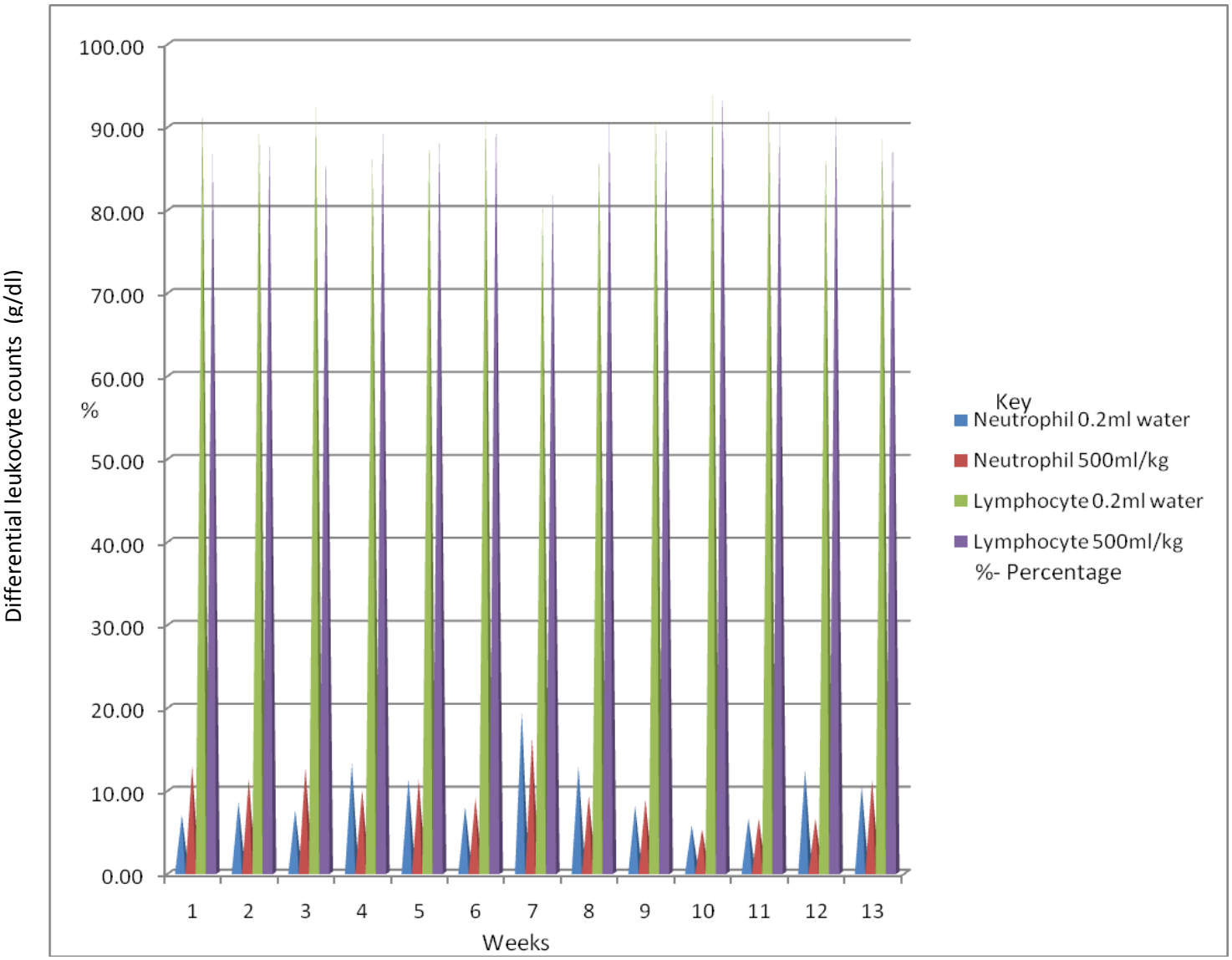


Figure 4.4: Differential leucocyte counts of broiler chickens treated with 500 mg/kg of *K senegalensis* extract for 14 days

4.5.3 Weight Gain

Birds treated with the extract (500 mg/kg) had statistically significant ($P<0.05$) increased weight gain when compared with the untreated control birds at the ninth week post treatment with the extracts of *Khaya senegalensis*. However, the increased weight gain was not statistically significant ($P>0.05$) by the twelfth week (Figure V).

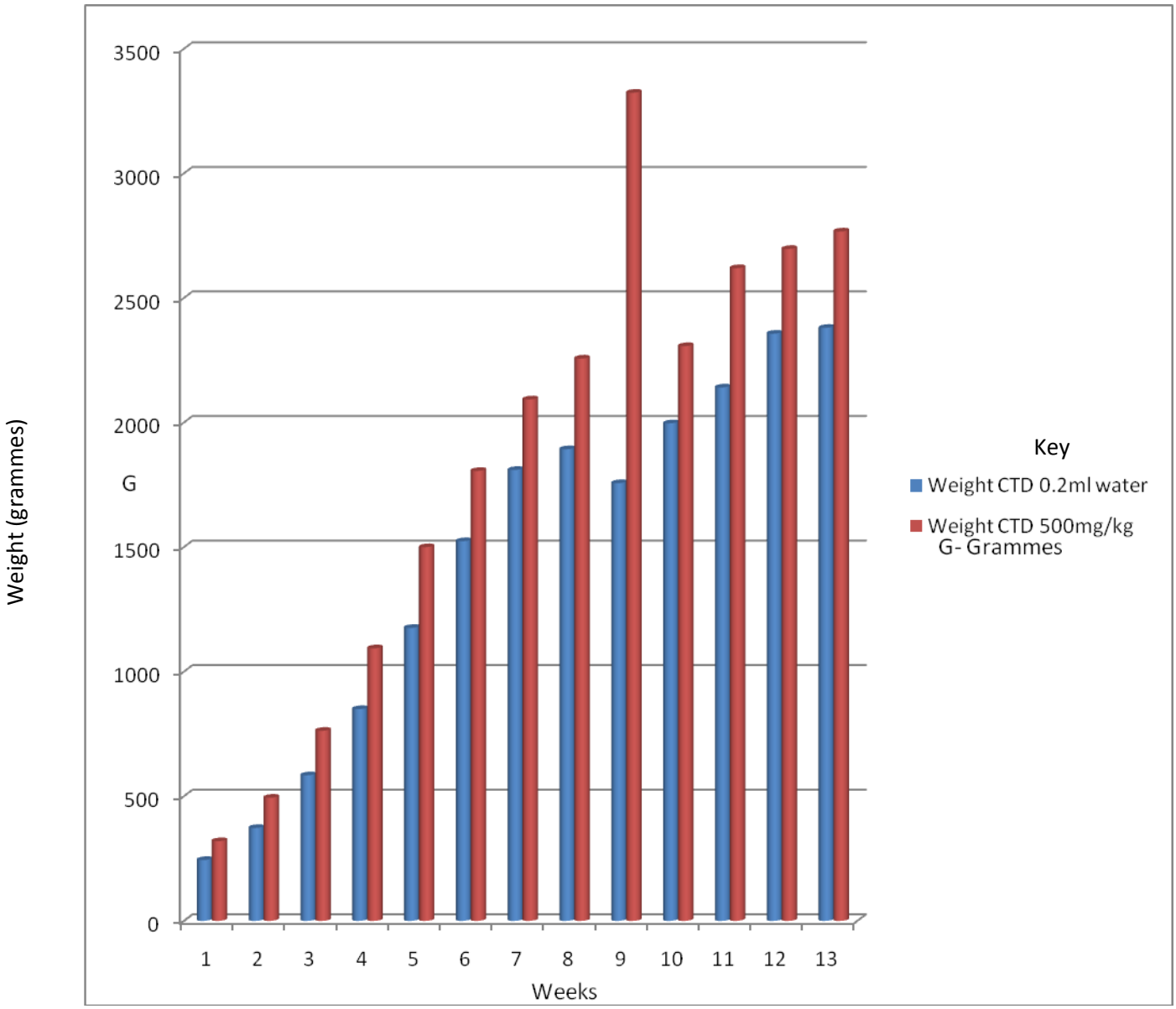


Figure 4.5: The effects of the leaf extract of *Khaya senegalensis* (500 mg/kg) administered for 14 days on the weight of broiler chickens

4.6 Comparison of the treatment of the experimentally infected birds with *Khaya senegalensis* and Amprolium

The experimentally infected birds began to shed oocysts in the faeces 6 days post infection. Pretreatment clinical signs such as bloody diarrhoea, bloody perineum, feet and feathers, depression and anorexia were observed. No signs were seen prior to the shedding of the oocysts. However, faecal examination revealed oocyst load of +++ in all the infected pens (A-E). Mortalities were recorded in all the infected 6 days post infection.

4.6.1 Gross Lesions

Gross lesions seen 7 day post infection revealed enlarged, bloody caeca, friable liver and the carcass was pale (Plates 4.18, 4.19 and 4.20). However one week post treatment, post mortem lesions seen in the caeca were all gone with the caeca of the extract treated birds and amprolium similar (Plates 4.21-4.24). The caeca of the uninfected untreated birds was distended with faecal material compared to that of the infected untreated group whose caeca were highly reduced in size compared (Plates 4.25 and 4.26). 12 birds comprising of 2 from each group A-F that were humanely sacrificed 13 days post treatment showed also no gross lesions in the carcasses.

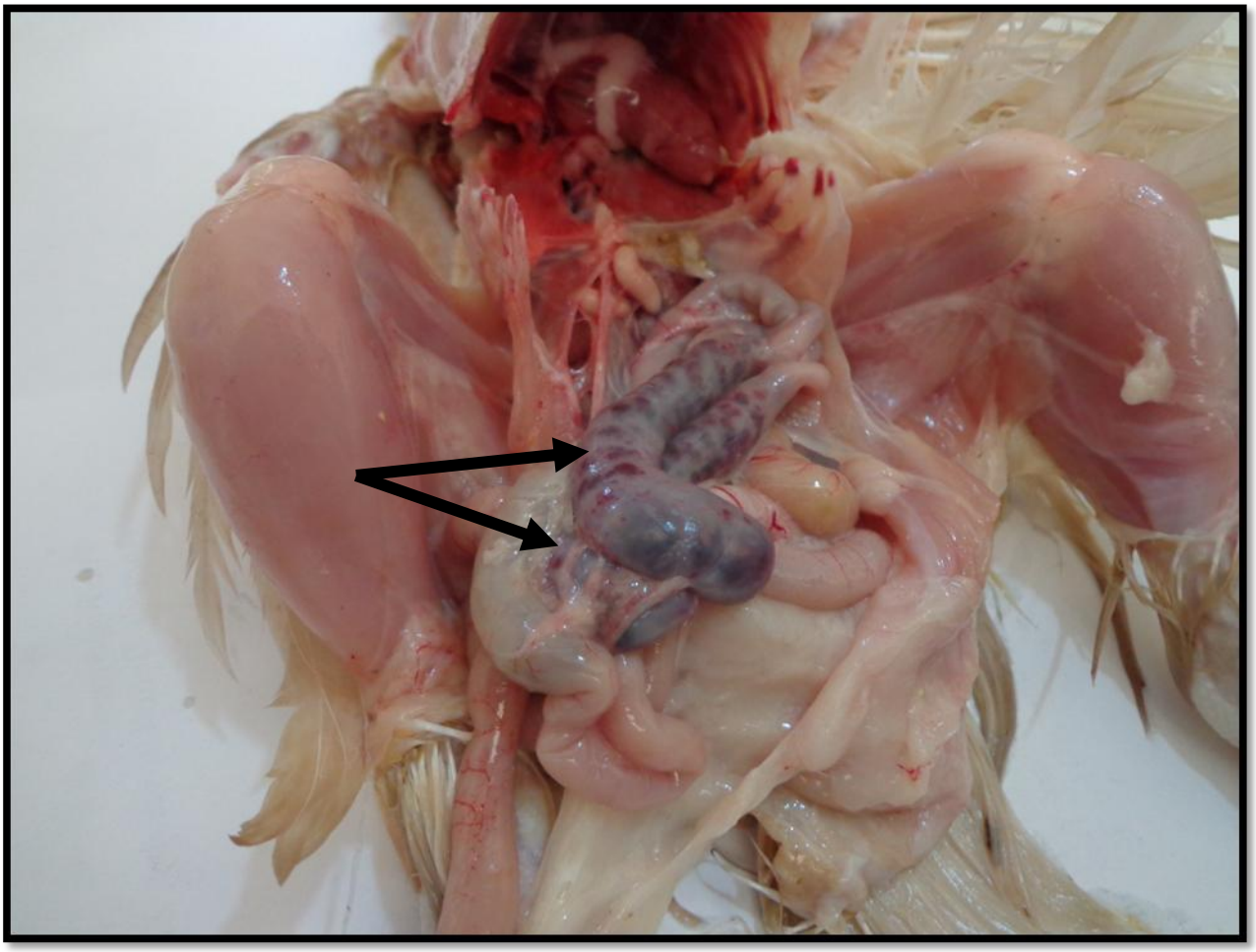


Plate 4.19: Gross lesion of *Eimeria tenella* infected broiler caeca distended with blood (arrows)

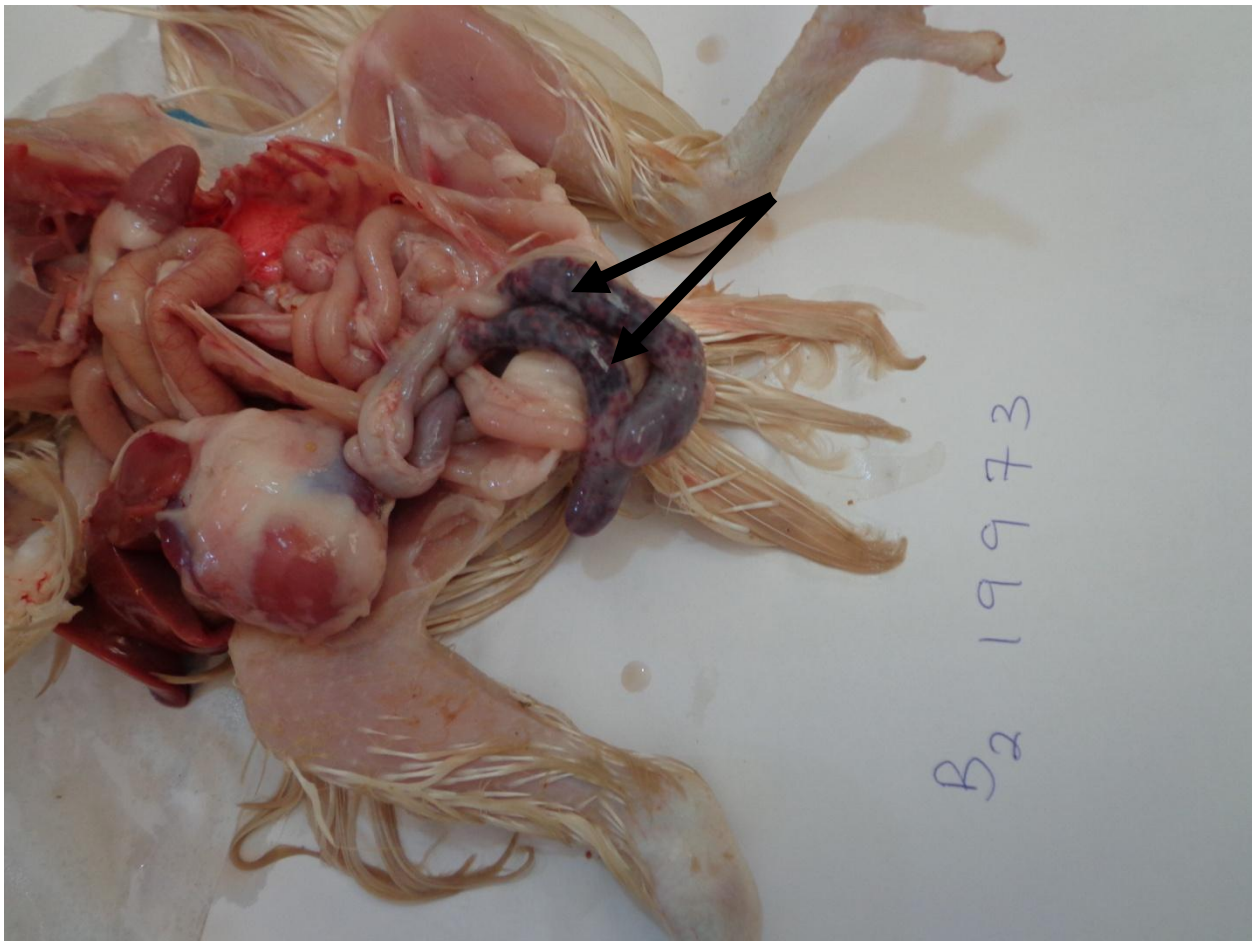


Plate 4.20: *In situ* appearance of *Eimeria tenella* infected broiler caeca distended with blood (arrows)

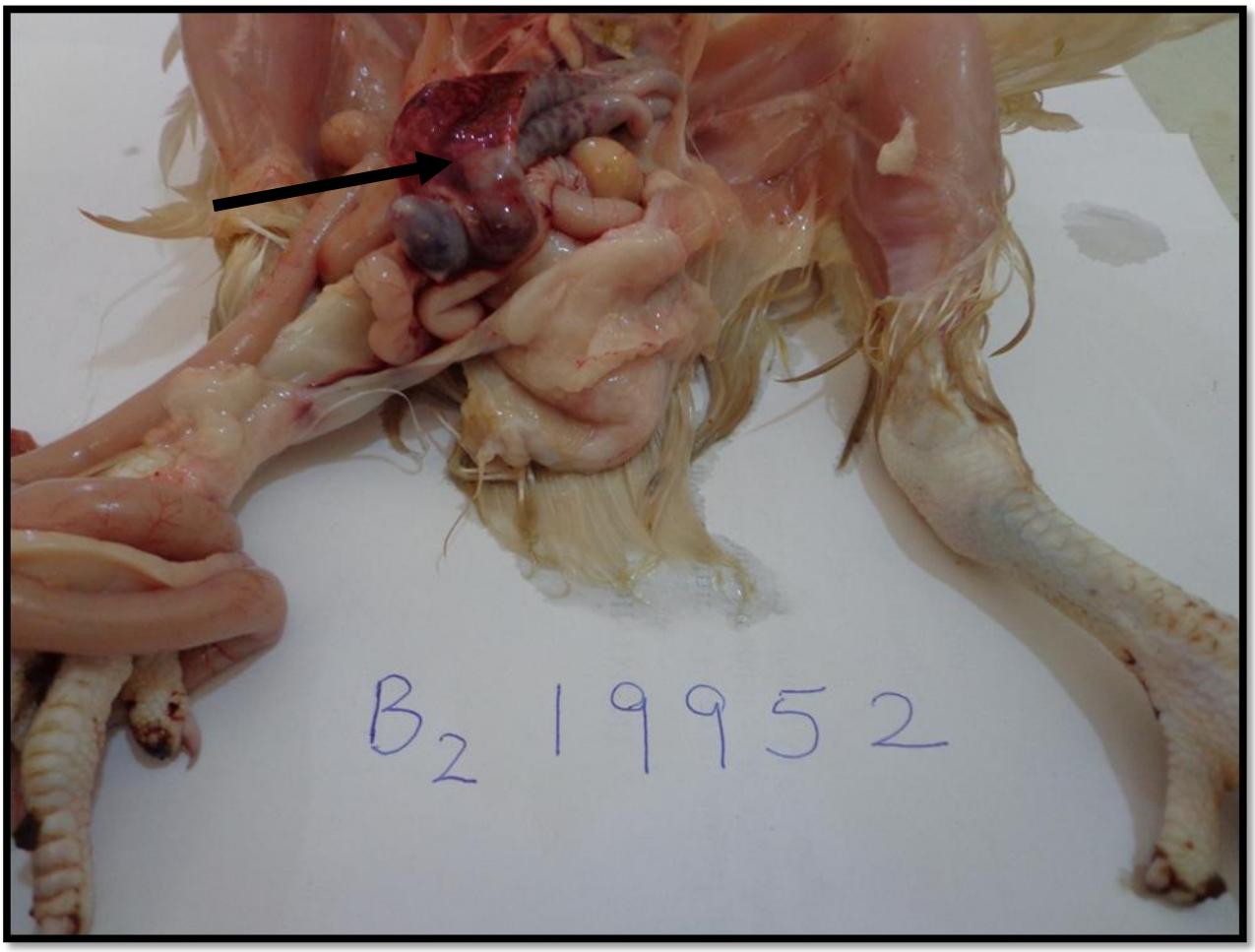


Plate 4.21: Bloody caecal contents of infected boiler chicken with *Eimeria tenella* (arrow)



Plate 4.22: Caeca of an *Eimeria tenella* infected bird one week post treatment with n-butanol fraction of the extract of *Khaya senegalensis* (11 mg/kg) filled with faecal contents (arrows)



Plate 4.23: Caeca of an *Eimeria tenella* infected bird one week post treatment with n-butanol fraction of the extract of *Khaya senegalensis* (33 mg/kg) (arrows)

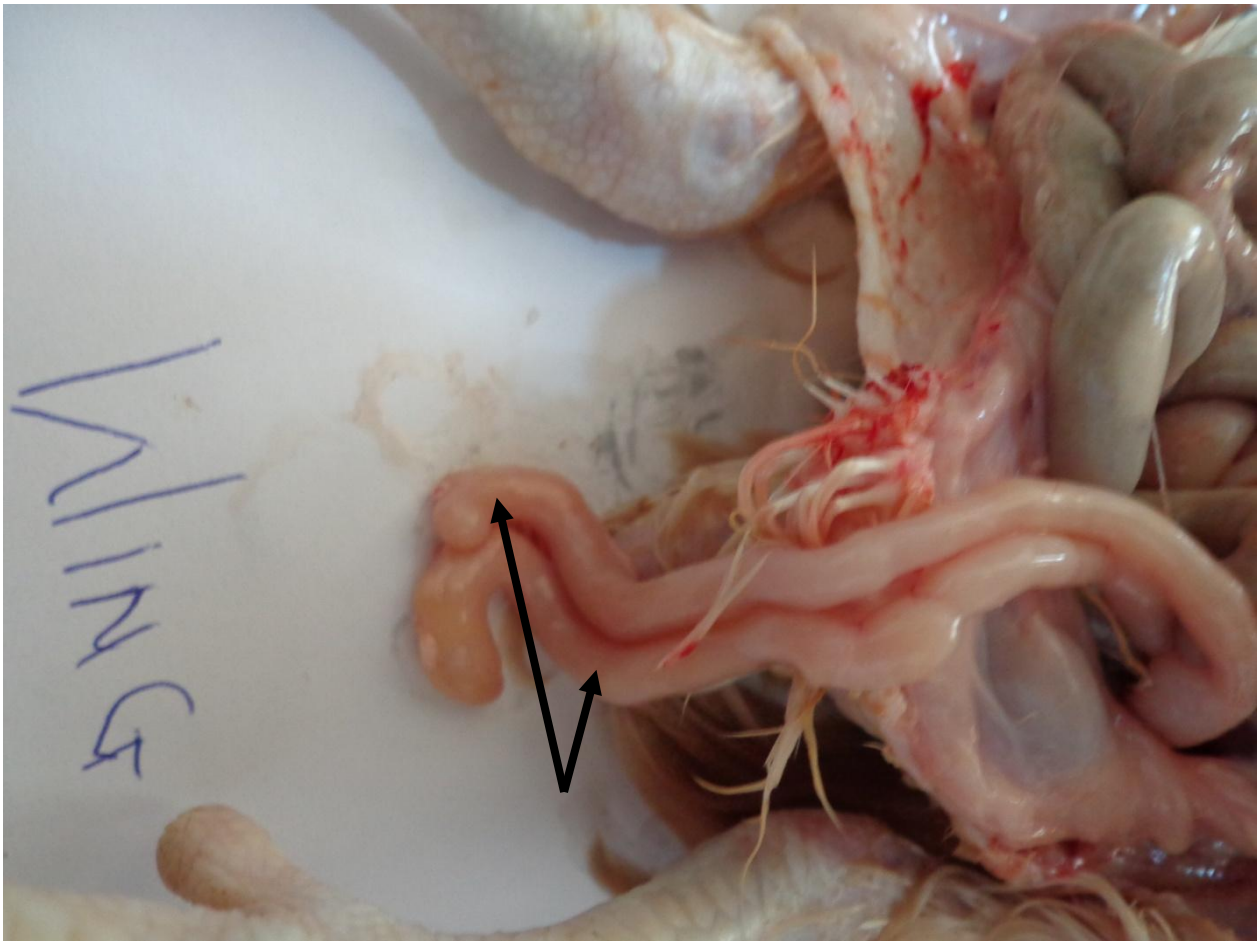


Plate 4.24: Caeca of an *Eimeria tenella* infected bird one week post treatment with n-butanol fraction of the extract of *Khaya senegalensis* (99 mg/kg) (arrows)



Plate 4.25: Caeca of an *Eimeria tenella* infected bird one week post treatment with Amprolium (1.2g/l) (arrows)



Plate 4.26: Shrunken caeca of *Eimeria tenella* infected non treated bird (positive control) one week post treatment with water (0.2 ml/ bird) (arrows)



Plate 4.27: Caeca of a non infected broiler chicken (negative control) one week post treatment with water (0.2 ml/ bird) filled with faecal contents (arrows)

4.6.2 Microscopic Examination of Caecum

Pre infection microscopic examination of intestinal scrappings revealed no parasitic infection. 7 days post treatment 11mg/kg (group A) revealed scanty schizonts and merozoites seen compared with the untreated infected group (group E) which had massive schizonts seen Plates 4.27 and 4.28). The group infected and treated with Amprolium (Group D) had few merozoites and schizonts seen (Plates 4.29). Similarly in group C, (99mg/kg) had merozoites and schizonts were seen. In 33mg/kg (group B) and negative control; un infected and untreated (group F) no parasites were seen.

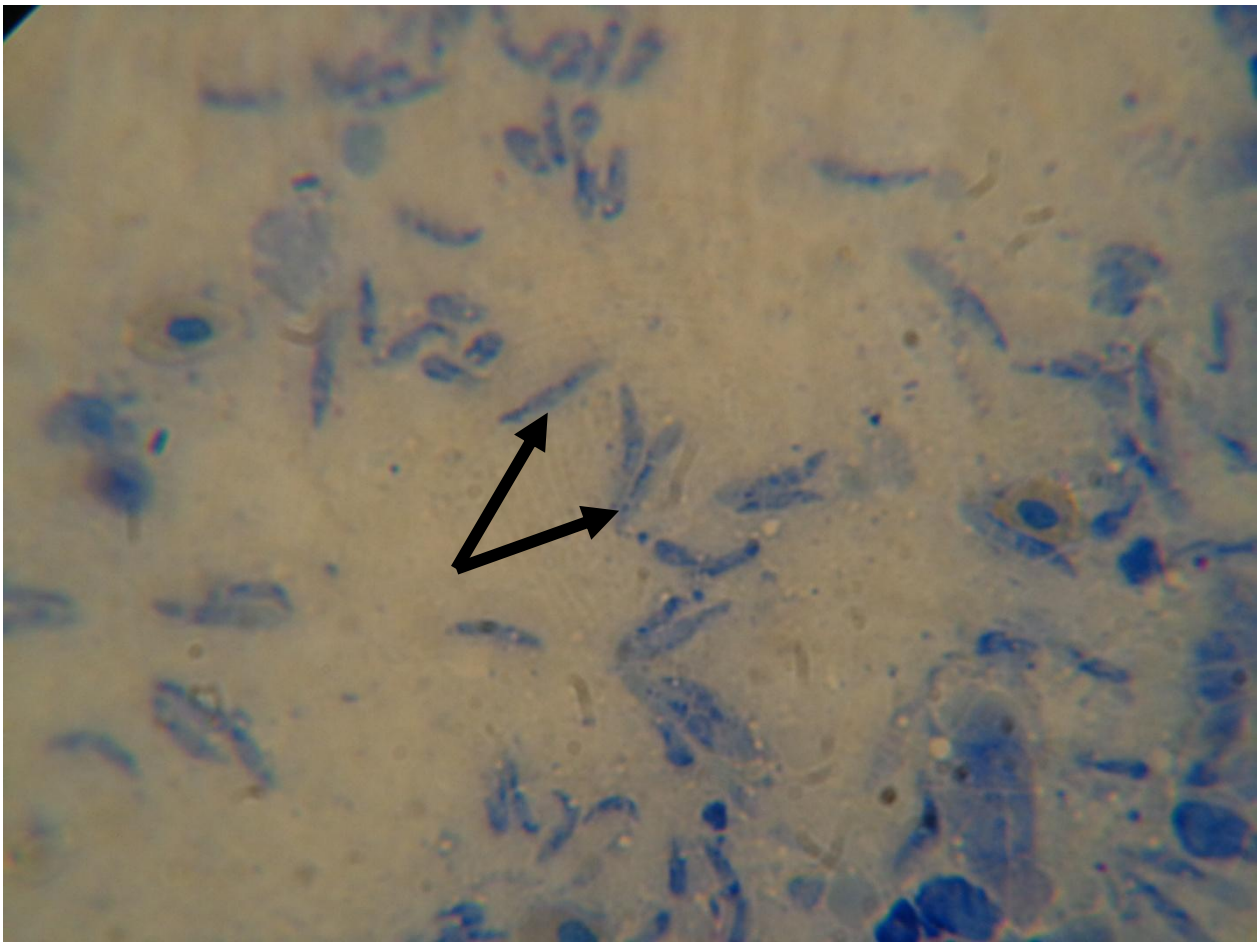


Plate 4.28: Photomicrograph of merozoites from caecal scrappings of infected un treated birds (arrows). Giemsa stain × 100 magnification

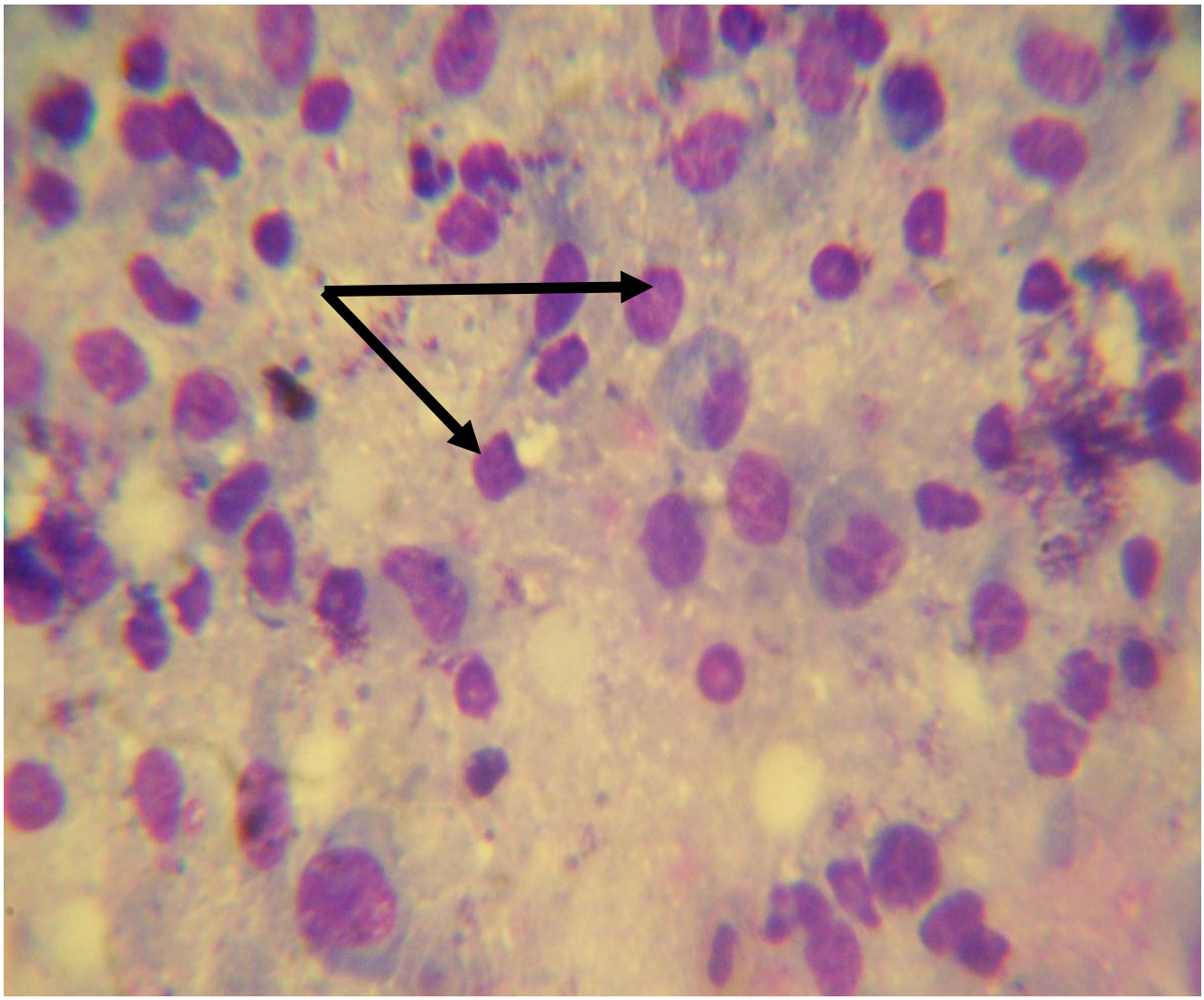


Plate 4.29: Photomicrograph showing schizonts from caecal scrappings of infected untreated bird (arrows). Giemsa stain × 100 magnification

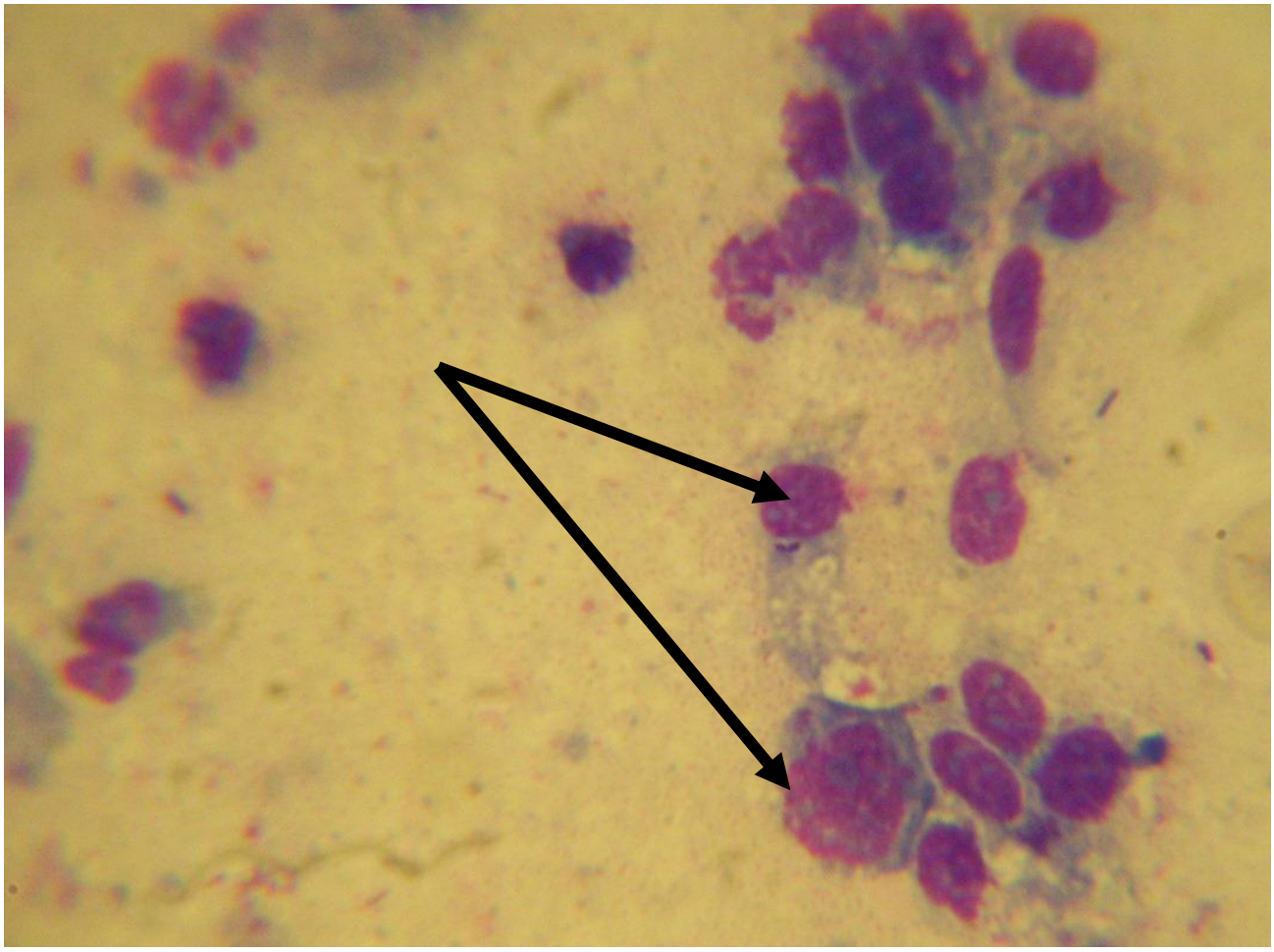


Plate 4.30: Photomicrograph showing scanty schizonts from caecal scrappings of infected and Amprolium treated bird (arrows). Giemsa stain × 100 magnification

4.6.3 Haematological values for experimentally infected broiler chickens treated with *Khaya senegalensis*

The differences between the means of the different groups were not statistically significant (P>0.05) for weeks 1, 2 and 4 though for one week post treatment, the mean PCV of the treated groups were slightly lower than the control, the lowest being in the Amprolium treated group (28.267±0.852 vs 29.588±0.800). Similarly the mean PCV of the infected untreated group was lower than the extract treated groups with highest PCV being in the 33mg/kg group (28.353±0.800 vs 30.579±0.57). For the 2nd week post treatment, the mean PCV of the treated groups were slightly higher than the control, the highest being in the Amprolium treated group (35.588±1.976 vs 29.333± 1.921). By the 4th week, the mean PCV of the extract treated and Amprolium treated groups were comparable to the control with highest PCV recorded in the Amprolium treated group (34.133±2.574 vs 31.941±2.418). This was also recorded for the infected untreated group. However, for week 3, there was statistically significant differences in the means when the group given Amprolium at 1.2g/L was compared with the group given the extract at 11mg/kg (P<0.05). Also there was statistically significant differences in the means of the PCV between the groups given 11mg/kg and the group infected but not treated (P<0.05). There was statistical differences in the means of PCV between the groups treated with 99mg/kg and the 11mg/kg (P<0.05). Also there were statistically significant differences between the groups treated with 11mg/kg of the extract and the uninfected group that was given 0.2ml of water. The 3rd week recorded the lowest PCV (24.667±1.531) among the extract treated groups in the 11mg/kg group and the highest in the 99mg/kg group (31.000±1.326) and this was lowest recorded throughout the period of study of the broiler chickens. The Amprolium treated group maintained higher PCV values after the initial fall at the first week (Table 4.11).

Table 4.11: Mean Packed Cell Volume of birds one to four weeks post treatment with n-butanol fractions of *Khaya senegalensis*

Treatment	PCV (%) (Mean ± SE) P<0.05			
	Weeks Post treatment			
	1	2	3	4
Extract 11mg/kg	28.875±0.825	30.917±2.352	24.667±1.531	32.071±2.664
Extract 33mg/kg	30.579±0.57	30.692±2.260	28.923±1.471	32.364±3.006
Extract 99/mg/kg	28.706±0.800	31.500±2.577	31.417±1.531	33.000±3.323
Amprolium 1.2g/L	28.267±0.852	35.588±1.976	32.467±1.370	34.133±2.574
Positive Control(infected, 0.2ml water)	28.353±0.800	31.667±2.352	32.222±1.768	33.375±3.524
Negative Control(infected, 0.2ml water)	29.588±0.800	29.333±1.921	31.000±1.326	31.941±2.418

Key:

PCV- Packed Cell Volume

SE- Standard Error

P<0.05- Statistically significant

The statistical analysis on the differential leukocyte count values showed no correlation when compared with the different levels of treatment (11mg/kg, 33mg/kg, 99mg/kg and the use of conventional drug such as Amprolium at 1.2g/L) .There was also no correlation with the infected untreated group and the uninfected and untreated group (the control) but when compared within each other there was a correlation. Among the extract treated birds, there was increased lymphocytic value (90.66±3.32 vs 90.52±3.46) as well as a decreased Haemoglobin count (9.86±2.19 vs 10.02±1.95) in the 11mg/kg group when compared with the negative control. Similarly, among the treated groups, the Amprolium treated group had the lowest lymphocyte value (89.40±4.05). The neutrophil value of the 11mg/kg group was the lowest (9.13±3.32) while the 33mg/kg had the highest values (9.62±3.77) compared to the control (9.19±3.39). The haemoglobin count of the treated groups had the 33mg/kg group with the lowest value (9.81±1.81) while the Amprolium treated group had the highest value (10.60±2.63) when compared to the negative control (10.02±1.95).The total protein values among the treated groups were slightly lower than the negative control, the lowest being in the Amprolium treated group (3.64±0.54) when compared to the control (3.69±0.48). The positive control had differential leucocyte count generally had values lower than the treated birds except the total protein (Table 4.12).

Table 4.12: Differential Leucocyte count of birds treated with the different levels of treatment with n-butanol fractions of *Khaya senegalensis*

Variable	**Differential Leucocyte Count (Mean ±STD) (P<0.05)**					
	11mg/kg	**33mg/kg**	**99mg/kg**	**Amprolium**	**+ve control**	**-ve control**
Neutrophil (%)	9.13±3.32	9.62±3.77	10.17±4.01	10.26±3.96	9.33±2.98	9.19±3.29
Lymphocyte (%)	90.66±3.32	90.19±3.77	89.48±4.34	89.40±4.05	90.41±3.01	90.52±3.46
Haemoglobin (g/dl)	9.86±2.19	9.81±1.81	10.09±2.02	10.60±2.63	9.99±1.94	10.02±1.95
Total Protein (g/dl)	3.68±0.60	3.68±0.51	3.65±0.50	3.64±0.54	3.77±0.48	3.69±0.48

Keys:
STD - Standard deviation
P<0.05- Statistically significant
+ve - Positive control
-ve - Negative Control

4.6.4 Weight

The pre-infection mean weight for group 2 (33mg/kg) was statistically significant (P<.0.05) when it was compared with Group 6 (negative control). Similarly, the positive control group showed statistically significant difference when compared with the uninfected group that was each administered only 0.2ml of water (P<0.05). When the different mean weights were compared with the different treatment groups(11mg/kg, 33mg/kg, 99mg/kg, Amprolium, positive control (given 0.2ml water) and the negative control (treated with 0.2ml water), there were no statistical significant difference (P>0.05) (Table 4.13).

Table 4.13: Pre-Infection mean weight (grammes) of the experimental Birds

Treatment	Weight (grammes) (Mean ± SE) P<0.05
Extract 11mg/kg	442.86±16.26
Extract 33mg/kg	490.00±16.87
Extract 99mg/kg	437.50±17.56
Amprolium 1.2g/L	430.00±15.17
+ve control (infected/0.2ml water)	485.00±19.24
-ve control (uninfected/0.2ml water)	374.18±14.75

Keys:

+ve- Positive

-ve - Negative

SE- Standard Error

P<0.05- Statistically significant

There was no statistically significant differences ($P>0.05$) in the mean weights in the first week when they were compared between the various treatment levels. In week 2, the mean weights for groups 1, 3 and Amprolium showed similar statistical significance ($P<0.05$) when they were compared with group 6 (negative control). There was statistical significance in the mean weights of the groups for the third week (1, 2, 3, 4 and 5) representing Extract 11mg/kg, Extract 33mg/kg, Extract 99mg/kg, Amprolium 1.2g/L and those administered only 0.2ml of water and the uninfected untreated group. Apart from group 1, there was no difference ($P<0.05$) in the mean weights between weeks 3 and 4 when compared with group 6(negative control) (Table 4.14).

Table 4.14: Mean weights (grammes) in *Eimeria tenella* infected birds at one to four weeks post infection treated with *Khaya senegalensis*

Treatment	Weight (grammes) (Mean ± SE) P<0.05			
	Weeks Post treatment			
	1	2	3	4
Extract 11mg/kg	459.29±20.36	495.00±32.78	759.29±42.54	924.29±48.21
Extract 33mg/kg	560.00±21.13	607.69±34.01	964.62±44.15	1175.38±50.03
Extract 99/mg/kg	531.67±21.99	570.00±35.40	948.33±45.95	1177.50±52.08
Amprolium 1.2g/L	495.00±24.67	543.33±31.66	854.00±41.10	1032.00±46.58
Positive Control(infected, 0.2ml water)	565.00±24.09	605.00±38.78	948.00±50.34	1155.00±57.05
Negative Control(infected, 0.2ml water)	546.47±18.48	754.71±29.74	1044.12±38.61	1197.06±43.75

Keys:

SEM- Standard Error

P<0.05- Statistically significant

4.6.4.1 Weight Gain

One post treatment, the weight gain between the different treatment groups showed no statistically significant differences ($P>0.05$). However, two weeks post treatment there was statistically significant difference in the mean weight gain when they were compared between the treatment groups. Group 1,2,3,4 and 5 were statistically significant when compared with group 6 ($P<0.05$). The gain in weight three weeks post treatment was marginal and statistically not significant ($P>0.05$) as seen in (table 4.9) There was statistically significant difference ($P<0.05$) in the weight gain of the infected birds treated with 11 and 33mg/kg of the extract and also the Amprolium treated group, four weeks post infection when compared with the uninfected control. However, the weight gains in the other treated groups were not statistically significant ($P>0.05$) (Table 4.15).

Table 4.15: Weight gain (grammes) in *Eimeria tenella* infected birds at one to four weeks post treatment with *Khaya senegalensis*

Treatment	Weight Gain (grammes) (Mean ± SE) P<0.05			
	Weeks Post treatment			
	Wk2–Wk1	Wk3–Wk2	Wk4–Wk3	Wk5–Wk4
Extract 11mg/kg	16.43±17.52	35.71±24.00	264.29±22.28	165.00±22.91
Extract 33mg/kg	70.00±18.18	47.69±24.91	356.92±23.12	210.77±23.78
Extract 99/mg/kg	94.17±18.92	38.33±25.93	378.33±24.06	229.17±24.75
Amprolium 1.2g/L	65.33±16.93	48.00±23.19	310.67±21.52	178.00±22.13
Positive Control(infected, 0.2ml water)	80.00±20.00	40.00±28.40	343.00±26.36	207.00±27.11
Negative Control(infected, 0.2ml water)	172.35±15.90	208.24±21.78	289.41±20.22	152.94±20.79

Keys:

WK- Week

SE- Standard Error

P<0.05- Statistically significant

4.4.4.2 Total weight gain

There was statistically significant difference when the mean total weight gain of group 1 (11mg/kg) was compared with group 6 (uninfected, 0.2ml water) ($P<0.05$). All others were not statistically significant ($P>0.05$) The weight gain in the 99mg/kg group was comparable to that of the negative control Also among the extract treated groups, there was a progressive increase in weight gain (Table 4.16)

Table 4.16: Mean total weight gain (grammes) of experimental birds for a four week period post treatment with n-butanol fraction of *Khaya senegalensis*

Treatment	Weight Gain (grammes) (Mean ± SE) P<0.05
Extract 11mg/kg	481.43±44.83
Extract 33mg/kg	685.38±46.53
Extract 99mg/kg	740.00±48.43
Amprolium 1.2g/L	602.00±43.31
+ve control (0.2ml Water infected)	670.00±53.05
-ve control (0.2ml Water uninfected)	822.94±40.69

Keys:

SE- Standard Error

P<0.05- Statistically significant

4.6.5: Observation of oocyst load and shedding by *E tenella* infected and extract treated birds

The oocyst output recorded for birds infected with *Eimeria tenella* was highest one week post infection with the 33mg/kg group producing the highest oocysts counts (29%). Among the treated group, the Amprolium treated birds recorded the lowest oocysts out put one week post treatment (5%). Similarly, the second week post treatment had the 11mg/kg (21%) and 33mg/kg (23%) extract treated infected groups with the lowest oocysts counts while the 99mg/kg had the highest (25%). However, two weeks post treatment, among the treated groups, the 33mg/kg and 99mg/kg both had the lowest oocysts counts (12%) comparable to the Amprolium treated group (10%). By the third week post treatment the 99mg/kg treated group was outstanding with the lowest oocysts counts (2%) and this was the lowest oocysts counts among the infected treated groups.The positive control maintained the highest oocysts output from the second week post infection (26%) rising to 64% by the fourth week post infection while the uninfected had no oocysts counts (0%) until the second week (1%) due to contamination (Tables 4.17 and 4.18).

Table 4.17: Experimental *Eimeria tenella* oocysts production one week post infection

Group	Doses	Oocysts production/gramme of faeces	% Oocysts production/g of faeces
A (Extract)	11mg/kg	64350	17
B (Extract)	33mg/kg	111000	29
C (Extract)	99mg/kg	68000	18
D (Amprolium)	1.2g/L	90200	24
E (Positive	0.2ml (water)	49600	13
F (Negative control)	0.2ml (water)	0	0
Total	--	383150	100

Table 4.18: Average weekly experimental *Eimeria tenella* oocysts production post treatment

Group	Doses	Average weekly oocysts production /gramme of faeces and Percentage production (%)

		Week 1(%)	Week 2(%)	Week 3(%)
A (Extract)	**11mg/kg**	***1,389,510 (21)**	**13,840 (17)**	**16,580 (18)**
B (Extract)	**33mg/kg**	**1,521,280 (23)**	***10,060 (12)**	**7,120 (8)**
C (Extract)	**99mg/kg**	***1,680,820 (25)**	***9,720 (12)**	***1,920 (2)**
D (Amprolium)	**1.2g/L**	**340,600 (5)**	***8,600 (10)**	***7,000 (8)**
E (Positive control)	**0.2ml (water)**	***1,748,849 (26)**	***39,420 (48)**	***59,140 (64)**
F (Negative control)	**0.2ml (water)** **-**	**10 (0)**	**470 (1)**	**800 (1)**
Total		**6,681,069 (100)**	**82,110 (100)**	**92,560 (100)**

4.6.6 Survival Rate of experimentally infected birds

The survival percentage was higher in the medicated groups compared to the infected un medicated group. Among the medicated groups, survival percentage was numerically higher in the 33mg/kg treated group while the least was in the 99 mg/kg treated group (Table 4.18).

Table 4.19: Survival rate of experimentally infected birds with *E tenella* and treated with different doses of *Khaya senegalensis*

Group	Drug	Dosages	No.of Birds	Pre-TM mortality	Post-TM mortality	Total mortality	Birds Alive	Pre-Survival	Post-Survival	% Survival	% Pre-Survival	%Post - Survival
A (Extract)	1	11mg/kg	20	3	1	4	16	17	16	80	85	80
B (Extract)	1	33mg/kg	20	3	0	3	17	17	17	85	85	85
C (Extract)	1	99mg/kg	20	4	2	6	14	16	14	70	80	70
D(Amprolium)	2	1.2g/L	20	1	1	2	18	19	18	90	95	90

E(Positive)	3	0.2ml	20	8	1	9	11	12	11	55	60	55
F (Negative control)	3	0.2ml	20	0	3	3	17	20	17	85	100	85

Key

TM – Treatment

4.6.7 Histopathological Lesions

The histopathological lesions observed one week post treatment was the sloughing of the mucosal surface into the intestinal lumen in the 11mg/kg treated group and desquamation of intestinal epithelium/villi with sloughing into the intestinal lumen in the infected untreated group (Plates 4.31 and 4.32). No significant findings were seen in both Amprolium treated and the uninfected untreated groups. However, 4 weeks post treatment, there was reduction and desquamation of the intestinal villi with intact intestine in the 99mg/kg treated groups (Plates 4.33). All other groups showed no significant findings.

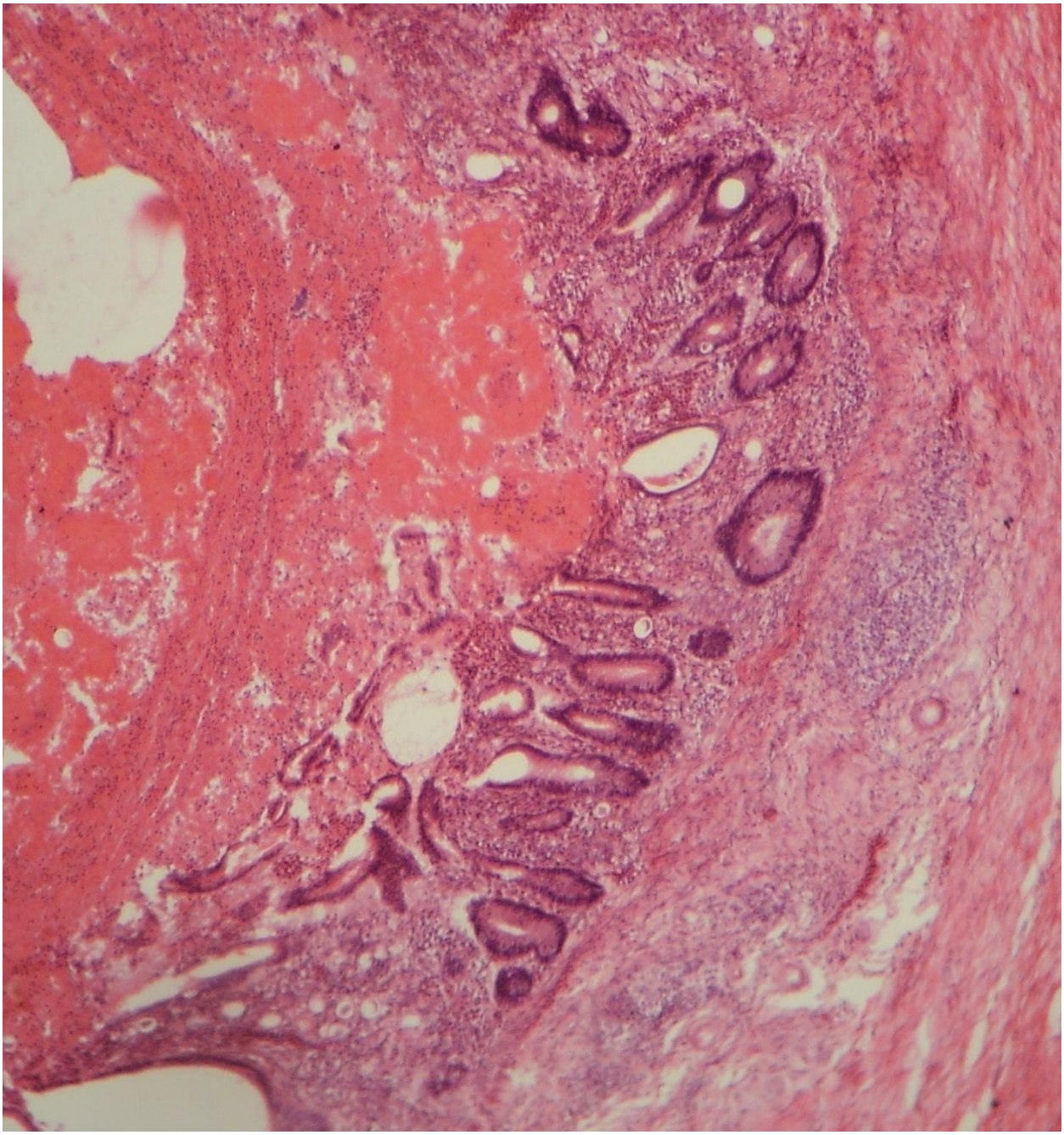


Plate 4.31: Sloughing of mucosal surface into lumen of intestine of infected and treated with extract (11 mg/kg). (H and E) × 40 magnification



Plate 4.32: Desquamation of intestinal epithelium/villi with sloughing into intestinal lumen of Positive control (infected untreated group) one week post treatment (arrow). (H and E) × 40 magnification



Plate 4.33: Reduced and desquamation of villi with intact intestines an infected and treated bird with 99 mg/kg four weeks post treatment (arrows). (H and E) × 40 magnification

Chapter Five

5.0 Discussion

The results of the chromatography showed that *Khaya senegalensis* has phenols and flavonoids which are secondary metabolites. Studies have shown that sources of natural antioxidants (Emami, 2007) are primarily plant phenolics such as flavonoids that exhibit antioxidant, antimicrobial, anticarcinogenicity and other biological activities (Aziman *et al*., 2012) and recent pharmacological studies have proven that flavonoids possess antiviral, antibacterial and anti inflammatory activities *in vitro* (Liu *et al*., 2015). They also have high antioxidant activity and may have anticoccidial effects (Ferreira *et al.*, 2010). Antioxidants which are defined as substances present at low concentration relative to the oxidizeable substrate which significantly delay or prevent oxidation of the substrate (Anyasor and Ogunwenmo, 2010; Okugbo and Oriakhi, 2015). Free radicals are inevitably produced in biological systems and also exogenously when they are in excess cause damaging effects on cells and antioxidants combat free radicals by intervening at any one of the three major steps of the free radical mediated oxidative processes viz initiation, propagation and termination (Kedare and Singh, 2011). Studies show that the human body does not synthesize overwhelming amounts of anti oxidants (Halliwell, 1990) and this indirectly can be related to poultry to compensate for the damaging effects of reactive oxygen species (ROS) and oxygen free radicals (Anyasor and Ogunwenmo, 2010). The production of synthetic antioxidants such as butylated hydroxyl toluene, gallic acid esters and tertiary butylated hydroquinone which have the potential to neutralize free radicals, they have been criticized due to possible toxic effects, low solubility along with moderate antioxidant activity (Kothari and Seshadri, 2010). Anti oxidant from plant sources are currently receiving

increasing attention due to their potential health benefits, availability and affordability (Omoregie *et al*., 2014). Also anti oxidant compounds such as flavonoids containing compounds which inhibit growth of pathogens and are least toxic to host cells are good candidates for new drug developments (Aziman, *et al*., 2012). This can be applied to *Khaya senegalensis* which can have its natural antioxidant compounds harnessed for therapeutic purposes due to its availability and affordability as well as its beneficial health implications. The greenish colouration obtained when Ferric chloride was sprayed on the plate is in agreement with Anyasor *et al*. (2010) in which he also obtained greenish colouration when ferric chloride was sprayed on flavonoids containing extracts. The result of the chromatography also implied the active ingredients were present mainly in the n-butanol and crude extract fractions. The high tendency of polyphenols to chelate metal ions may contribute to their antioxidant activity by preventing redox-active transition metals from catalyzing free radical formation and may be responsible for the greenish colouration seen when Ferric chloride was sprayed on the extract (Aude and Edwards-Levy, 2011). The antioxidant activity of *Khaya senegalensis is* not only dependent on the phenolic content but also the phenolic acid component (Horax and Islam, 2005). Polyphenolics possess hydroxyl and carboxyl groups able to bind to metal ions bearing strong positive charges such as iron III and copper II (Aude and Edwards-Levy, 2011) and this may directly contribute to the antioxidant activity and is a key determinant of their scavenging and metal chelating activities (Elmastas *et al*.,, 2007).

The large R_f values obtained indicate that non polar compounds were mainly present in the n-butanol, aqueous and crude extract fractions. This implies that the phenolic compounds present in *Khaya senegalensis* were non polar and so would not easily dissolve in polar solvents example of which is water. Also the speed with which the spots easily moved up the plate suggested they

had less attraction for the stationary phase. This is in agreement with Cannell (1998) who reported that non-polar compounds have less affinity for the stationary phase and will move comparative very quickly up the plate and therefore have relatively larger R_f values.

The aqueous extract of *Khaya senegalensis* had the least anti sporulation activity due to the presence of sporulated oocysts as against the n-butanol suspension for *K senegalensis.* This suggests that the anticoccidial activity was absent in the aqueous fraction of the plant leaves extract. Traditionally *Tamarindus indica* has been said to have anti protozoal activities Gefu *et al*. (2000), however, the sporulation medium (2.5% Potassium dichromate) did enhance sporulation of the oocysts. Lack of inhibition of sporulation by *Guiera senegalensis* and *Tamarindus indica* as seen in these studies may be attributable to the absence of anticoccidial factor in their extracts. The n-butanol fraction of *K senegalensis* and the aqueous fraction of *Tamarindus indica* extracts had similar effects to those obtained using Amprolium which was considered as the standard. Amprolium which has been proved to reduce oocyst sporulation (Arakawa *et al*., 1981) also completely inhibited the sporulation of the oocysts in this study. Consequently the mechanism of action of n-butanol of *K.senegalensis*, aqueous fractions of T indica and Amprolium may show some similarities.

Clinical signs and gross lesions associated with the maximum tolerated dose seen in this study are in agreement with Nwosu *et al*. (2011).

The high survival rate of 100% in the five medicated groups indicated the high safety margin of *K senegalensis* even at a dose of 891mg/kg. This is in agreement with Usman *et al*. (2011) who recorded no mortality in a study using *K senegalensis*. Abdel-Wareth *et al*. (2014) also reported

similar findings when experimental rabbits were fed with *K senegalensis* leaves and no mortality was recorded.

None of the haematological values were significantly changed when the extract was given even at 891mg/kg. The increase in the Packed cell volume and the haemoglobin count and were near normal and this could be related to the active ingredients present in the extract. This finding is in agreement with Ali *et al*. (2011) who had similar results when aqueous extract of *K senegalensis* were given to rats, though at higher doses it was said to have a destructive effect on the bone marrow. The increase in the lymphocytic values of the treated groups confirm the reports of Brisibe *et al*. (2008) and Sani *et al*. (2013) on the positive effects of natural flavonoids on the immune system of different species which focus on mainly the T lymphocyte stimulation and increasing blood lymphocytes and the lymphocytosis is indicative of inflammation (Abdel Wareth *et al*., 2014).

The progressive weight increase in both the medicated and un medicated group shows the ability of the extract to improve weight gain as reported by Nwosu *et al*. (2011). Among the treatment groups the differences in weight gain was recorded with highest in the 11mg/kg and the lowest in the 891mg/kg groups may be associated with the hepatotoxic and nephrotoxic effect at higher doses. The hepatoprotective, hepatotoxic and nephrotoxic effects of *K senegalensis* leaves have been reported by Adebayo *et al*. (2003), Abubakar *et al*. (2010) and Ali *et al*. (2011). Thus at lower doses *K senegalensis* can be used as growth promoter due to the increase in weight. Abdel-Wareth *et al*. (2014) also reported similar findings in an experiment when rabbits were fed with a diet containing 35% *K senegalensis* leaves, the test animals were said to have increased weight gain and carcass traits, however the reverse was the case when a diet containing 65% of the leaves was used and the rabbits had lower weights than the former group. The weight gain in the

11mg/kg group might also be associated with the antioxidant compounds present in the extract (Ali *et al*.,2011) and the nutrient values of the leaves of *K senegalensis* which are said to be rich in gross energy and crude protein (Abdel-Wareth *et al*., 2014).

The histopathological lesions seen are in agreement with Abdel-Wareth *et al.* (2014) who reported some degree of nephrotoxicity among the experimental rabbits fed diets with *K senegalensis* leaves. He also discovered that the leaves had some effects on vital organs which are dose-dependent. This was noticed at high doses where there was lymphocytosis which was indicative of inflammation.

The study confirmed the safety of the extract of *K senegalensis* even at high doses. Nwosu *et al*. (2011) reported that even when *K senegalensis* was administered at a dose of 2000mg/kg only mild clinical signs were seen. The gross lesions however, showed that 500mg/kg given over a two weeks period had effects on the internal organs of the birds. This may mean that although *K senegalensis* may be tolerated physiologically, there may be some injurious effects associated with the administration of higher doses. This is in agreement with Onu *et al.* (2013) who reported similar findings in a study on rats that the severity of lesions seen was concentration dependent being more severe in those rats given the highest concentration of the extract. It should therefore not be given at a dose above 500mg/kg.

The packed cell volume and the haemoglobin count been higher than the control are in agreement with Ali *et al*. (2011) and Usman *et al*. (2011) who also had a similar finding when aqueous extract of *K senegalensis* was given to rats. Lymphocytosis was noticed across the treated groups. This could be as a result of the effect of the extract on the internal organs. Dkhil *et al*. (2012) reported that lymphocyte increase could be a prominent response of body tissues

facing injurious impact. The rising nature of post treatment weights revealed the impact of chronically administering the 500mg/kg of the extract. This implies that though it causes weight gain as earlier established, it has some effect on vital organs and such can only be administered for a short duration.

The clinical signs and lesions observed in the birds challenged with *Eimeria tenella* such as bloody diarrhoea, high mortality and distended caeca with bloody faeces and mucous debris on the sixth day experimental infections are in agreement with the previous studies (Patra *et al*., 2010; Zaman *et al*., 2012; Meskerem *et al*., 2013).

The high oocyst production obtained among the infected groups one week post infection is in agreement with Myung-Jo. (2014) where the peak production of oocyst production for a comparative study on *E tenella, E acervulina* and *E maxima* showed that *E tenella* and *E maxima* both had peak productions on the 7^{th} day. Also the irregular oocyst production observed in this study is in agreement with Myung –Jo. (2014) who had similar results. The higher mean oocyts count in the infected untreated group than the treated group indicated that the extract had some inhibitory effects on oocyst production. This however, depends on the dose of the extract given. This is again in agreement with Myung-Jo. (2014) who stated that the inherent difference in reproductive potential is high for *E tenella* which are dependent on some factors such as inherent potential to produce in a non-immune host, immunity or resistance of the host, nutrition and strain differences of the host. Among the extract medicated group the high oocyst production by the 11mg/kg group (17%) 2 weeks post treatment suggests the dose was not adequate to prevent oocyst production while the 99mg/kg had lower oocyst production (12%) 2 weeks post treatment and 2% three weeks post treatment which was comparable to the group treated with normal dose of Amprolium (10%) and 8%, two weeks and 3 weeks post treatment respectively and thus was

the effective dose. This result is in agreement with Nwosu *et al*. (2011) who reported that in a study conducted, *K senegalensis* had some anticoccidial effect mediated by reduction in oocyst production.

The presence of schizonts and merozoites seen in caecal scrappings showed that reproductive activities of the parasites were not completely halted even after treatment. It also shows that the endogenous stages were not susceptible to the extracts at all doses although the population in the medicated infected group were lower than those seen in the infected un medicated groups. This is in agreement with Al-FIfi, (2007) who suggested the phenolic contents of extracts possessed anti inflammatory properties which might act in the caecal epithelium cell protection which is detrimental to the coccidia reproductive activities. However, the uninfected untreated group had no parasite found in them.

The survival percentage was higher in the medicated groups compared to the infected un medicated group. Among the medicated groups, the survival rate was numerically higher in the 33mg/kg treated group while the least was in the group treated with 99mg/kg. The inverse trend in the survival rate and the dose of the extract can also not be easily explained as the expectation should be higher dose, higher survival rate. However, from observation on the histopathological lesions from the birds dosed with *K senegalensis* in the maximum tolerated dose experiment, the deaths might be related to the effect of the extract on vital organs at a higher dose which were given repeatedly for 5 days. The higher survival rate at lower doses could be as a result of the therapeutic effect of plant which is known to have phenolic compounds which are antioxidants and could have played a role in the survival of the birds as they have been identified in helping to block the action of/or inactivate lipid free radicals or by preventing the decomposition of hydroperoxides into free radicals which have been implicated in several stresses related to

gastrointestinal injuries (Pourmorad *et al*., 2006; Sani *et al*., 2013). This potential mechanism makes the diverse group of phenolic compounds an interesting target in search for health beneficial phytochemicals (Akinpelu *et al*., 2010).

The infected groups had slightly lower PCV even after treatment with the extracts than the uninfected or Amprolium treated group. This is in agreement with Ogbe *et al*. (2010) who reported a slight drop in the PCV and Hb counts in *Eimeria tenella* infected birds. A mean PCV of 19% at 6 days post infection is observed as the minimum for the survival of birds (Conway *et al*., 1993). In this experiment the lowest PCV was 24.66% at the 3rd week post infection in the 33mg/kg treated group. The reason being deduced for the PCV of the extract medicated groups not falling excessively might be due to the beneficial therapeutic activity of the plant *K senegalensis*. Usman *et al*. (2011) reported in a study that *K senegalensis* caused appreciation of the PCV of *E tenella* infected birds. Meskerem *et al*. (2013) in a study reported the reduction in the PCV of birds infected with *E tenella*. This finding reflects an anaemic condition with depressed metabolism due to tissue damage and immune responses in coccidial infected birds (Hirani *et al*., 2007). Anaemia characterized by decreased PCV and Hb is the most common erythrocyte abnormality in birds. Birds with PCV less than 35% are generally considered anaemic which is caused by blood loss through the gastrointestinal tract (Meskerem *et al*., 2013). Wide ranges of normal haematologic values have been reported by other authors (Ogbe *et al*., 2003; Iheukwumere *et al*., 2006). However, fluctuations in the haematological values of avian blood are normal phenomenon and in most instances, the variations in haematological values may depend on the physiological state of the birds (Islam *et al*., 2004).

The findings from the haematological analysis which revealed a lymphocytosis in all the infected groups .is in agreement with Patra *et al*. (2010) who suggested this might be due to

induction of the immune response in the infected birds due to increased lymphopoiesis. This is considered as a first step in defense mechanisms to fight infections. A similar observation was by Perez – Carbajal *et al*. (2010) also in a study where he reported leukocytosis due to mostly lymphocytosis. This might be precipitated by the invasion of the intestinal epithelium by the *Eimeria* sporozoites resulting in inflammation and subsequent initiation of the immune response and massive infiltration of macrophages, granulocytes and lymphocytes into the lamina propria (Usman *et al*., 2011; Meskerem *et al*., 2013) with lymphocytes in particular, the CD +4 T cells which act as inducer for an effective immune response (Bessay *et al*., 1996; Usman *et al*., 2011). There's increasing evidence that cell mediated immunity plays a major role in resistance to infection as T-lymphocytes appear to respond to coccidial infection through both cytokine production and a direct cytotoxic attack on infected cells (Yun *et al*., 2000).

The mean weight of the infected, treated group was statistically higher than the infected untreated group both medicated infected and un medicated infected groups and is in agreement with El-Abasy *et al*. (2003), Abbass *et al*. (2008) and Usman *et al.* (2011), who reported in a study higher weight gain in the treated group than the infected untreated group after challenge with *E tenella* oocysts. Also in a similar study Nwosu *et al*. (2011) reported weight gain in birds that had coccidial challenge and were treated with *Khaya senegalensis*. The higher weight gain seen 2 weeks post treatment in the 11mg/kg treated and the negative control could be as a result of the antioxidant compounds present in the extract as similar result was obtained in the maximum tolerated dose study. The loss in weight in both the treatment groups and the positive control 2 weeks post treatment could be as a result of inflammatory immune responses which divert energy from growth which may affect the weight gain (El-Abasy *et al*., 2003). *Eimeria* parasites invades the cells of the intestine producing enteritis and diarrhoea resulting in disability

to absorb dietary nutrients through the disruption of the integrity intestinal mucosa and this leads to loss of weight in the infected birds (Al-Idreesi *et al.*, 2013). Fetterer and Allen (2001) also in a study to determine *E tenella* effect on plasma and muscle 3-methylhistidine(3MH) reported that muscle breakdown which was assessed by plasma and muscle levels of 3MH was elevated during the acute stage of *Eimeria tenella* infection and was most likely associated with anorexia which was due to the infection.

The histopathological lesions observed in the infected birds in this study are consistent with those associated with *E tenella* infection in which the parasite induced very severe lesions including severe villous atrophy and fusion of the villi (McDougald and Reid, 1997; Khadim, 2014). The most pathogenic stage of *E tenella* is mainly due to the second generation schizonts which cause extensive tissue damage, bleeding, disruption of caecal glands and the destruction of the mucosa and the muscularis layer (McDougald and Fitz-Coy, 2008). The lesions still observed in the 99mg/kg group 4 weeks post treatment shows the irreversible damages done to the intestine by the *E tenella* parasite even though physically the birds appeared to have overcome the infection.

Chapter Six

6.0 Summary

- The n-Butanol fraction of the *Khaya senegalensis* extract contains phenols and flavonoids which have antioxidant effects.
- The result of the phytochemistry also implied the active ingredients were present mainly in the n-butanol and crude extract fractions.
- The large R_f values obtained indicate that non polar compounds were mainly present in the n-butanol, aqueous and crude extract fractions.
- The *in vitro* study showed that the n-butanol fraction of the *Khaya senegalensis* extract (100mg/kg) inhibits sporulation of *E tenella* oocysts.
- The n-butanol fraction of *K senegalensis* and the aqueous fraction of *Tamarindus indica* extracts had similar effects to those obtained using Amprolium which was considered as the standard.
- The maximum tolerated dose experiment revealed that the high survival rate of 100% in the five medicated groups indicated the high safety margin of *K senegalensis* even at a dose of 891mg/kg.
- None of the haematological values were significantly changed when the extract was given even at 891mg/kg. However, there was increase in the lymphocytic values of the treated groups.
- The progressive weight increase in both the medicated and un medicated group shows the ability of the extract to improve weight gain. Among the treatment groups the differences in weight gain was recorded with highest in the 11mg/kg and the lowest in the 891mg/kg

groups. Thus at lower doses *K senegalensis* can be used as growth promoter due to the increase in weight.

- The histopathological lesions observed show that the leaves had some effects on vital organs which are dose-dependent.
- In the Chronic toxicity study, there were no deaths recorded in the treatment groups. The gross lesions however, showed that 500mg/kg given over a two weeks period had effects on the internal organs of the birds.
- In this case unlike the Maximum tolerated dose (MTD), the packed cell volume and the haemoglobin count of the treated group were higher than the control. However lymphocytosis which was noticed across the treated groups was similar to what was obtained for MTD
- Clinical Disease and Therapeutic effects of the extracts showed clinical signs and lesions in the birds challenged with *Eimeria tenella* as bloody diarrhoea, high mortality and distended caeca with bloody faeces and mucous debris on the sixth day experimental infections.
- The high oocyst production was obtained among the infected groups on day 8 post infection.
- The n-butanol fraction of *Khaya senegalensis* at a dose of 99mg/kg two weeks post treatment reduced oocysts counts by 12% which was comparable to the standard drug (Amprolium;10%).
- The higher percentage of the mean oocyts count in the infected untreated group than the treated group indicated that the extract had some inhibitory effects on oocyst production.

Among the extract medicated group the high oocysts counts by the 11mg/kg group suggests the dose was not adequate to prevent oocyst production

- Amprolium was highly efficacious both *in vivo* and *in vitro* studies.
- The presence of schizonts and merozoites in caecal scrappings showed that reproductive activities of the parasites were not completely halted even after treatment. It also shows that the endogenous stages were not susceptible to the extracts at all doses although the populations in the treated infected groups were lower than those seen in the infected untreated groups.
- The survival percentage was higher in the treated groups compared to the infected un medicated group. Among the treated groups, the survival rate was numerically higher in the 33mg/kg treated group while the least was in the group treated with 99mg/kg.
- The infected groups had slightly lower PCV even after treatment with the extracts than the uninfected or Amprolium treated group. In this experiment the lowest PCV was 24.66% at the 3rd week post infection in the 33mg/kg treated group. The findings from the haematological analysis which revealed a lymphocytosis in all the infected groups
- The mean weight of the infected, treated group was statistically higher than the infected untreated group. The higher weight gain was seen 2 weeks post treatment in the 11mg/kg treated and the negative control.
- The histopathological lesions observed such as desquamation, reduction and sloughing of the intestinal villi into are consistent with those associated with *E tenella* infection in.

Conclusion

Results from this study demonstrated that the n-butanol fractions of the crude methanol extract of *Khaya senegalensis* contained phenols and flavonoids that were very effective against experimental *Eimeria tenella* oocysts *in vitro* (100%) at a concentration of 100mg/ml. The *in vivo* study revealed an anticoccidial activity of 12% at a dose of 33mg/kg and 99mg/kg two weeks post treatment in broiler chickens which was comparable to Amprolium (a conventional anticoccidial) at a dose of 1.2g/L. However, the phenols at a dose of 99mg/kg three weeks post treatment in infected birds reduced oocysts count by 2%. The *in vivo* model was also found to be a useful tool for rapid screening of anticoccidial activity of plant preparations against *Eimeria* parasites.The presence of phenolic compounds is an interesting and developing new area of research which has potential of solving some of the health related issues concerning oxidants in both humans and poultry in particular. A quality control extraction of *Khaya senegalensis* and the isolation of bioactive compounds could be a promising alternative to conventional anticoccidials for the treatment of avian coccidiosis in the future.

6.1 Recommendation

- It is recommended that extract of *Khaya senegalensis* can be given at a dose of 11mg/kg as a natural growth promoter.
- Also anticoccidial *in vitro* activity of *K senegalensis* as it relates *in vivo* anticoccidial activity is an interesting area for more research.
- The anti oxidant compounds such as flavonoids and phenols which were identified in the leaves of *Khaya senegalensis* good candidates for new drug developments.

- Detailed studies are needed to isolate, characterize and evaluate the active components and the mechanism of action of the identified active principles in *Khaya senegalensis* leaves as well as other parts of the plant.

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Appendices

Appendix A



Plate I: ***Khaya senegalensis is*** **in its natural habitat**

Appendix B



Plate II: Soxhlet Apparatus (Quick Fit Corning Ltd. A division of Stafford, England) used for defatting the leaves of the plants

Appendix C



Plate III: Cold maceration extraction of *Khaya senegalensis* in 70% methanol

Appendix D

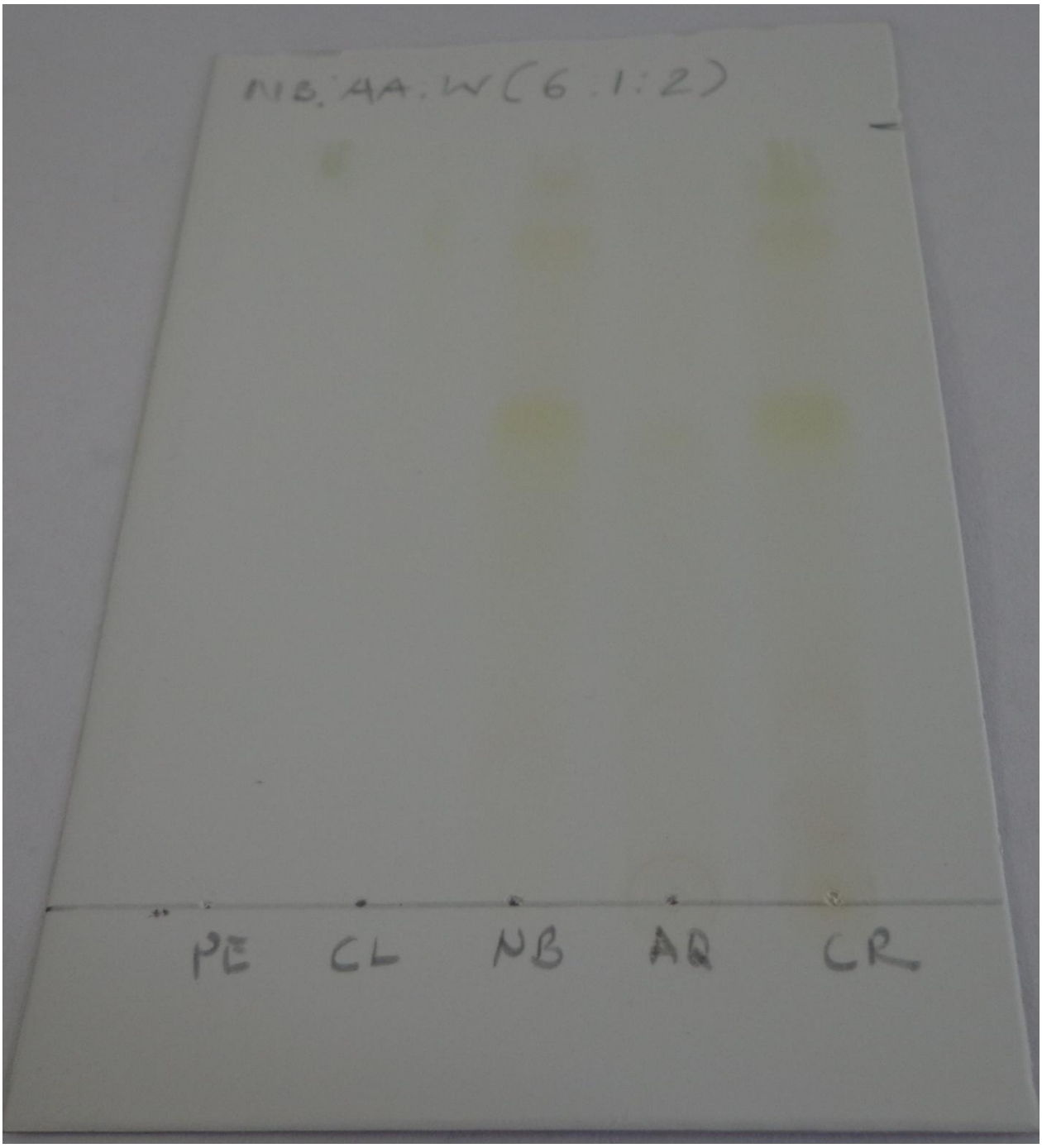


Plate IV: Pre-sprayed Thin Layer Chromatography plate

Mathematical formula for calculating the Retention Factor as well as time taken to run the TLC experiment

R_F = Retention Factor

R_F $\frac{distance\ travelled\ by\ spot\ divided}{distance\ travelled\ by\ solvent\ front}$

$R_F = \frac{S_a}{S_{of}}$

Time started = 10.25am

Time stopped = 11.55am

Time for TLC to run = 90 minutes

Appendix E

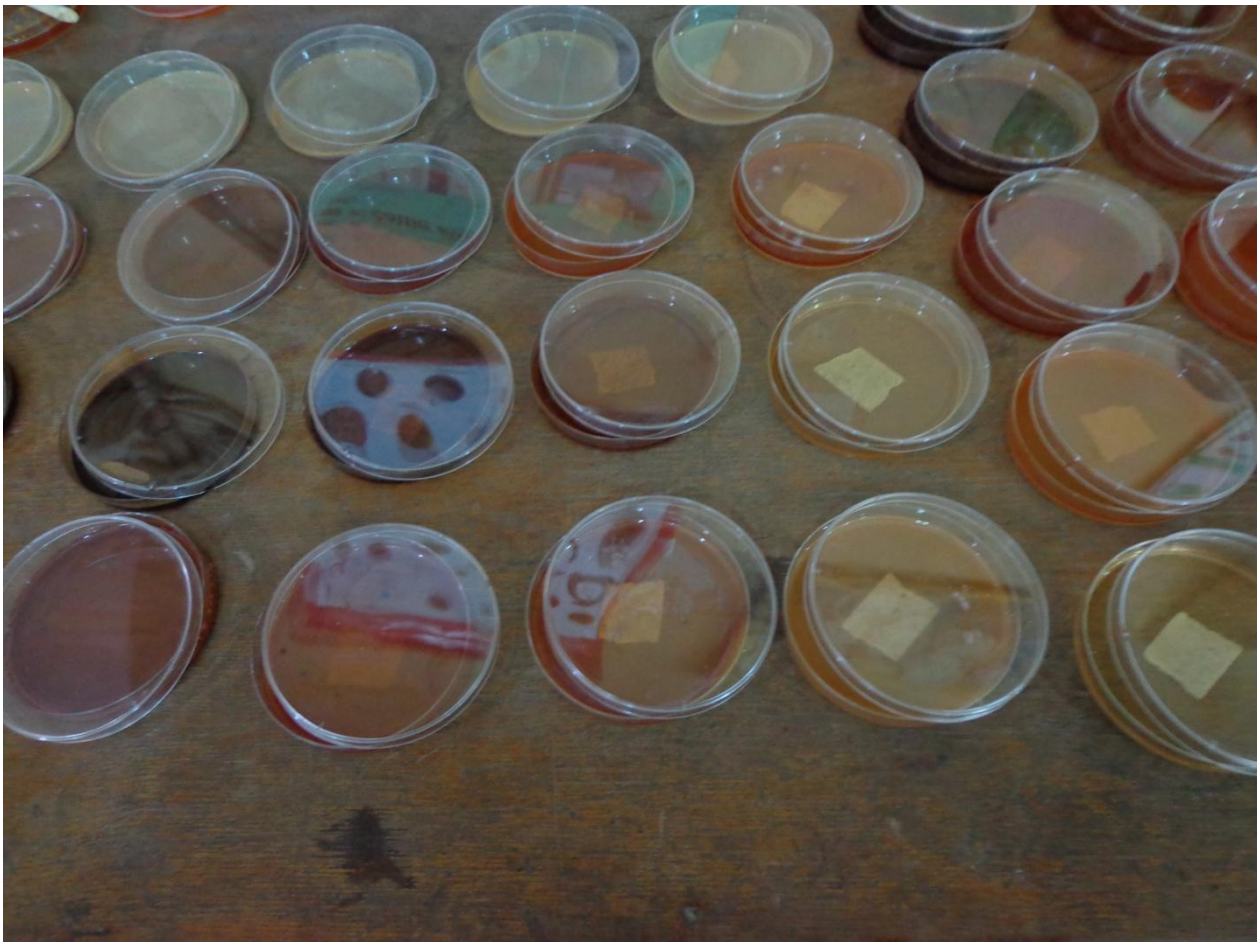


Plate V: ***In vitro*** **experiment setup with the suspension of the n-butanol fractions of the three Plant Extracts (*Khaya senegalensis, Guiera senegalensis* and *Tamarindus indica*) and *Eimeria tenella* oocysts**

Appendix F

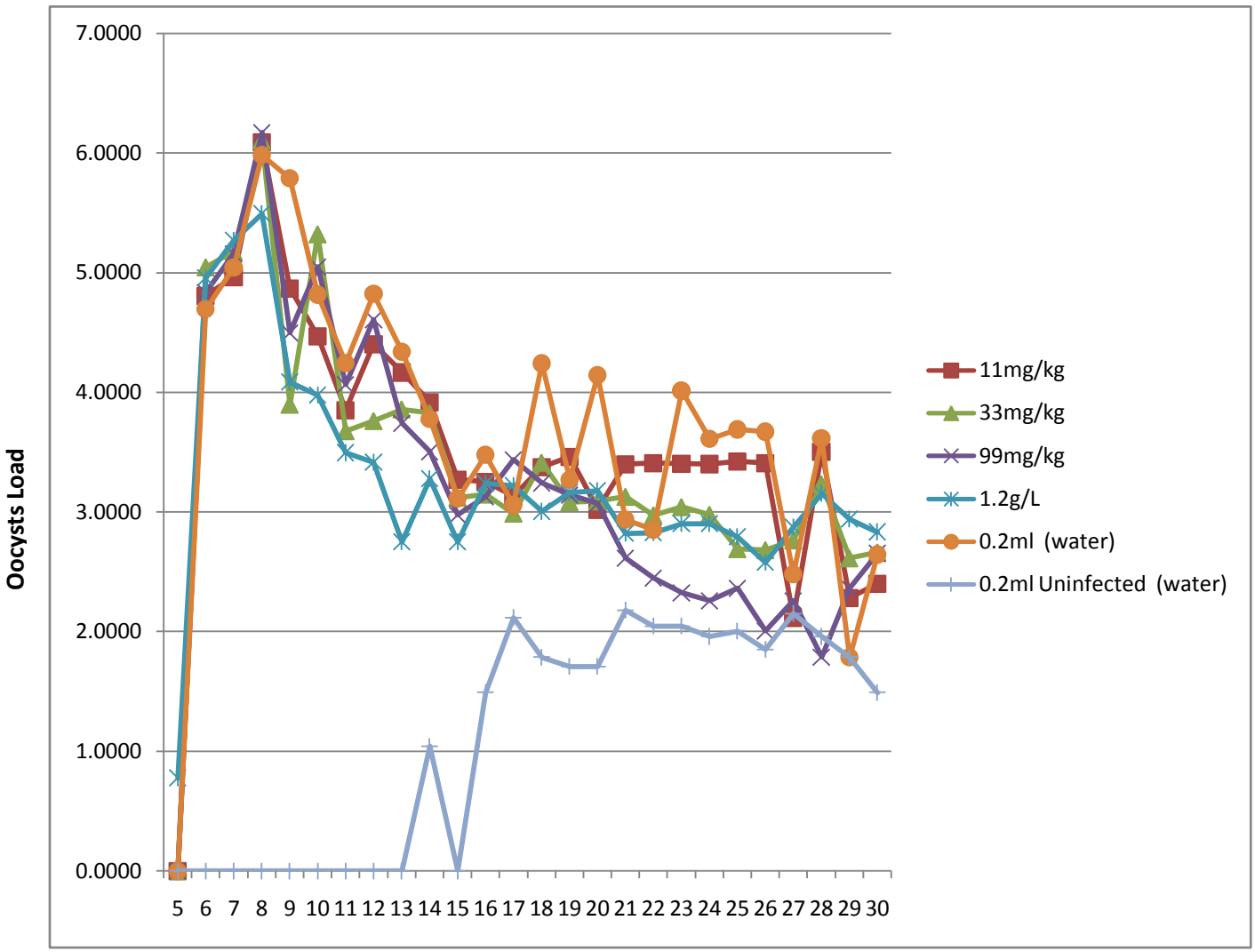


Figure I: Oocyst Load in Birds challenged with *Eimeria tenella* over a 4 weeks study period post treatment

Appendix G



Plate VI: Birds used for maximum tolerated dose experiment

Appendix H



Plate VII: Birds in chronic toxicity study

Appendix I



Plate VIII: Experimentally Infected broiler chickens with *Eimeria tenella* with bloody diarrhoea and blood stains on their feathers (arrows).