ANTHELMINTIC EFFICACY OF CRUDE METHANOL EXTRACT OF *DONNETIA TRIPETALA* G. BAKER FRUITS WITH ITS VARIOUS CHEMICAL FRACTIONS IN MICE EXPERIMENTALLY INFECTED WITH *HELIGMOSOMOIDES BAKERI*

BY

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NOVEMBER, 2015

Declaration

I declare that the work in this dissertation entitled **"Anthelmintic Efficacy of Crude Methanol Extract of *Donnetia tripetala* G. Baker Fruits with its Various Chemical Fractions in Mice Experimentally Infected with *Heligmosomoides bakeri*"** has been carried out by me in the Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Name of student	Signature	Date

Certification

This dissertation entitled "ANTHELMINTIC EFFICACY OF CRUDE METHANOL EXTRACT OF *DONNETIA TRIPETALA* G. BAKER FRUITS WITH ITS VARIOUS CHEMICAL FRACTIONS IN MICE EXPERIMENTALLY INFECTED WITH *HELIGMOSOMOIDES BAKERI*" by Khadijah SHUAIBU meets the regulations governing the degree of Master of Veterinary Pharmacology of the Ahmadu Bello University, Zaria and approved for its contribution to knowledge and literary presentation.

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Dedication

To Almighty Allah for His bounties and infinite mercies.

Abstract

The effects of *in vitro* and *in vivo* anthelmintic activity of crude methanol extract (CME), hexane extract (HE), ethylacetate extract (EAE), butanol extract (BT) and aqueous methanol extract (AME) of *Donnettia tripetala* fruits on mice experimentally infected with *Heligmosomoides bakeri* were evaluated. The fruits were washed dried, pounded and the powder was extracted with methanol in a soxhlet apparatus. Fifty grams of the crude methanol extract (CME) was partitioned with hexane (HE), ethylacetate (EAE) and butanol (BT). The extracts were subjected to preliminary phytochemical screening and acute toxicity test (LD_{50}). The *in vitro* anthelmintic studies involved the evaluation of egg hatch inhibition and larvicidal activities of the extracts. Different concentrations (0.07, 0.15, 0.3, 0.6, 1.25 and 2.5 mg/ml) of the CME, HE, EAE, BT and AME were prepared in distilled water. Two hundred microlitres of each concentration were incubated with the eggs and larvae of *H. bakeri* contained in 0.2 ml solution in 96 well microtiter plates and incubated at room temperature for 48 and 24 hours, respectively. Distilled water (0.2ml) and albendazole (0.07, 0.15, 0.3, 0.6, 1.25 and 2.5 mg/ml) were used as untreated and treated controls, respectively. In the *in vivo* trial, 85 mice infected with *H. bakeri* were randomly allocated into 17 groups of 5 mice each. Groups 1-3, 4-6, 7-9, 10-12, 13-15 were treated with CME, HE, EAE, BT and AME, respectively at doses of 200, 400 and 800 mg/kg. Groups 16 and 17 were treated with albendazole (10 mg/kg) once and distilled water (5 ml/kg) respectively, and served as treated and untreated controls. All treatments were administered orally on the 16^{th}, 17^{th} and 18^{th} day post infection. Anthelmintic activity was assessed by comparing the faecal egg count reduction rates and the worms recovered from the treated groups to the non- treated group. The extraction with methanol yielded 0.07 % of the powder. Furthermore, fifty grams of the crude methanol extract yielded 24.87 g (49.7%) of HE,

2.11 g (4.2%) of EAE, 3.69 g (7.4%) of BT and 16.80 g (3.4%) of AME on partitioning. Phytochemical screening revealed the presence of carbohydrate, cardiac glycosides, saponins, steroids, triterpenes, flavonoids, tannins and alkaloids in the CME, EAE, BT and AME. The HE however did not contain saponins. Acute toxicity study of the CME in mice showed that the extract was relatively non-toxic and safe at a dose of ≤ 5000 mg/kg. At 2.5 mg/ml the CME, EAE, AME and BT inhibited the hatching of *H. bakeri* eggs by 93.5, 92.4, 87.9 and 52.4%, respectively. Similarly, CME, EAE, AME and BT caused death of the larve of *H. bakeri* by 100, 92.6, 85.1 and 77.8%, respectively. However, HE did not produce significant *in-vitro* anthelmintic activity when compared with distilled water (untreated control). In the *in vivo* anthelmintic trial, the CME, HE, AME, BT and EAE at a dose of 800 mg/kg reduce fecal egg count by 100, 99, 98, 80 and 65%. Similarly, CME, EAE, AME, BT and HE caused deparasitization of 93, 93, 88, 88 and 82%, respectively, in mice infected with *H. bakeri* which is significant ($p<0.05$). The study concluded that *D. tripetala* fruit is safe and non toxic when administered acutely and posses anthelmintic activity.

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List of Abbreviations

ABU	Ahmadu Bello University
ABZ	Albendazole
AME	Aqueous methanol extract of *D. tripetala* fruits
ANOVA	Analysis of variance
BT	Butanol extract of *D. tripetala* fruits
CME	Crude methanol extract of *D.tripela* fruit
DW	Distilled water
EAE	Ethylacetate extract of *D. tripetala* fruit
ELISA	Enzyme Linked Immunosorbent Assay
EPG	Egg per gram
EPGR	Egg per gram reduction
FAO	Food and Agricultural Organisation
FECRT	Faecal egg count reduction test
FEC	Fecal egg count
HE	Hexane extract of *D. tripetala* fruits
IC_{50}	Concentrations of the extracts that inhibited 50 % of eggs from hatching
LC_{50}	Concentrations of the extracts that killed 50 % of the larvae
LD_{50}	Median Lethal Dose
nACh	n-Acetyl Choline
NaCl	Sodium Chloride
PMWC	Post mortem worm count
PTZ	Pentylenetetrazole
PCV	Packed Cell Volume
SEM	Standard Error of Mean

WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

Parasitic diseases have been noted as one of the major constraints to livestock productivity (Biu *et al*., 2006) causing enormous economic losses through morbidity and mortality (Waruiru *et al*., 2001). The direct losses caused by these parasites are attributed to acute illness and death, premature slaughter and condemnation of some animal parts at meat inspection. Indirect losses include the diminution of productive potential such as decreased growth rate, weight loss in young growing calves and late maturity of slaughter stock (Hansen and Perry, 1994).

Infections with parasitic helminths represent a significant economic and welfare burden to global livestock industry. The increasing prevalence of anthelmintic drug resistance means that current control programmes are costly and unsustainable in the long term (Van Dijk *et al*., 2010). Another factor of increased disease and production loss due to helminths is treatment failure, which is being reported even more frequently. Sustainable control of helminth infections requires detailed knowledge of these factors. There is a need to devise new, sustainable strategies for the effective control of helminthoses (Jackson and Miller, 2006) as frequent and widespread use and misuse of current control methods has resulted in the emergence of resistant parasite populations, such that anthelmintic resistance is now a major global problem, (Kaplan, 2004), and is the greatest threat to the sustainable control of helminthoses (Familton *et al*., 2001; Sangster and Dobson, 2002; Sutherland and Leathwick, 2010).

Traditional medicinal practices are known to be an important component of everyday life (Bussmann and Sharon, 2006). It has been a major aspect of the sociocultural

heritage in Africa for hundreds of years even before the advent of conventional medicine (Okigbo and Mmeka, 2008). Therefore its use in both developed and less developed countries, recognition and development of herbal medicine offer treatment methods that are more environmentally friendly, since they tend to be less toxic, produce fewer unanticipated side effects and apparently do not trigger anthelmintic chemoresistance (Sujon *et al*., 2008). The use of plants in healthcare is even much higher particularly in areas with little or no access to modern health services (Saeed, *et al*., 2004).

Donnettia tripetala is commonly known as pepper fruit and it is locally called "*mmimi*" and "*igberi*" in Igbo and Yoruba languages of Nigeria. The plant is widely distributed in tropical countries and it belongs to the family Annonaceae. In Nigeria, the plant is used for the traditional treatment of different diseases (Ejechi *et al*., 1999). The fruits, leaves, bark and roots of the plant possess strong pepperish and pungent taste and are popularly used as spices and condiments (Ejechi and Akpomedaye, 2005). The leaves are commonly used by the local herbalists in combination with other medicinal plants to treat various ailments including fever, infantile convulsion, typhoid, cough, worm infestation, vomiting, and stomach upset (Oyemitan, 2006). The plant also has mild laxative, antihyperglycemic, antibacterial and antifungal properties (Anaga and Asuzu, 2010).

H. bakeri is a standard experimental model used in testing anthelmintics of most nematodes of veterinary importance (Githiori *et al*., 2003). It is a trichostrongylid (Spurlock, 1943) and was originally called *Nematospiroides dubius* in the early 20th century, but later named *H. polygyrus*. The parasite is easily maintained in the

laboratory in white mouse (*Mus musculus*). The worm is presently called *H. bakeri* (Behnke *et al*., 1991) to distinguish it from *H. polygyrus sensu stricto*, the dominant intestinal nematode of wood mice in western Europe, which does not infect laboratory mice (*Mus musculus*) without the aid of an immunosuppressant.

1.1 Statement Of Research Problem

Helminthosis is one of the most important challenges militating against livestock production in the tropics often causing severe losses. Chemotherapy still remains the means of controlling helminthosis however the high cost of anthelmintics to small farmers, resistance to available anthelmintics and residue problems in meat and milk consumed by humans further complicates the use of chemotherapy (Akhtar and Ahmed, 1992).

Traditional medicinal plants are still used in the treatment of many diseases in developing countries (Rates, 2001; Tandon *et al*., 2011; Ngarivhume *et al*., 2015). However, many of the active ingredients in such plants are not known (Baker *et al*., 1995; Thomas, 1995).

1.2 Justification

In the face of rising cost of chemotherapy, increasing development of drug resistant parasites and tissue residues of chemotherapeutic agents, limits the use of chemical agents for the control of helminth parasites (Kudi and Myint, 1999; Gasbarre *et al*., 2001). Plant-based drugs are cheap and relatively safe (Kumara, 2001). It is therefore important to scientifically evaluate the potentials of plants and plant products used for ethnoveterinary medicine in Nigeria with a view of finding new therapeutic substances of natural origin that are effective and with low or no toxic effect to man and animals (Guarrera, 1999).

The use of plants and plant extracts as a possible source of new anthelmintics is currently receiving much interest (Adamu *et al*., 2012). *D. tripetala* a predominant plant in south-eastern Nigeria has been used to treat a lot of ailments in traditional herbal medicine especially worm infestation with little or no information on its anthelmintic efficacy.

Bioactive compounds isolated from plants could serve as lead compounds for the development of other useful pharmacological agents (Rates, 2001; Balunas and Kinghorn, 2005).

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study is to determine the anthelmintic effects of *D. tripetala* fruits in mice experimentally infected with *H. bakeri*.

1.3.2 Specific objectives

The specific objectives of the study are to investigate the:

- phytochemical constituents present in *D. tripetala* fruits
- egg hatch inhibition rates and larvicidal effects of *D. tripetala* fruits against *H. bakeri in vitro*
- *in vivo* anthelmintic effects of *D. tripetala* fruits in mice experimentally infected with *H. bakeri*

1.4 Null Hypothesis

Extract and fractions of *D. tripetala* fruits do not possess anthelmintic effect against *H. bakeri*

CHAPTER TWO

LITRATURE REVIEW

2.1 *Donnetia tripetala* G. Baker (Pepper Fruit)

D. tripetala (G. Baker) is an important indigenous plant of west tropical Africa (Etukudo, 2000), which has the synonym of *Uvariopsis tripetala* (G. E. Schatz). It is a native of Nigeria, Ivory Coast and Cameroon. It has its common name as the 'pepper fruit' in English. The plant is locally called "*mmimi*", "*nkaika*", "*Imako*" and "*Igberi*" in Igbo, Ibibio, Urhobo and Yoruba languages of Nigeria (Ejechi *et al*., 1999). *D. tripetala* belongs to the kingdom: *Plantae*; phylum: *Magnoliophyta*; class: *Magnoliopsida*; Order: *Magnoliales*; family: *Annonaceae*; genus: *Donnettia* (Kenfack *et al*., 2003). Species: *Donnettia tripetala* (Etukudo, 2000). The parts used traditionally include; the leaves, fruits, seeds, roots and stem.

2.1.1 Plant description

D. tripetala is a small to medium size woody shrub which reaches a height of about 12-18 m and girth of 0.6 m with a dense compact crown (Burkill, 1985). The wood is soft, white coloured and prone to termite attack. It has a fibrous bark which has a strong scent. The leaves are 3 – 6 inches long by 1.5–2.5 inches broad, elliptic to ovate, shortly acuminate and broadly connate to round at the base. The flowers are light brown outside, reddish inside and usually in small clusters on the young or older wood. The fruit is green at first, turns yellow and later red when ripe with finger-like carpel constriction between the seeds. The young leaves and fruit when ripe has a peppery and spicy taste when chewed (Burkill, 1985).



Figure 2.1: The tree of *D. tripetala* in its natural habitat showing the leaves, fruits and stem.

2.1.2 Domestic uses of *D. tripetala*

The fruits and leaves are commonly used as a spice, seasoning or natural flavour which is added to prepared food such as meat, sausage, stew, soup and vegetables (Ejechi and Akpomedaye, 2005). The bark of the tree is mixed with food to create variation in the taste and flavour of different foods. The wood is a good source of fuel wood (Duguma *et al*., 1990). The matured fruits constitute the main edible portion and are chewed in different forms (fresh green, fresh ripened red, black dry fruit and dry seed) because of its characteristic spicy tastea and; serve as a mild stimulant to the consumer (Ndukwu and Nwadibia, 2006; Oyemitan *et al.*, 2006). Its various parts are commonly used as spices and condiment (Oyemitan *et al*., 2008).

2.1.3 Traditional uses of *D. tripetala*

The leaves are used in folk medicine for the treatment of fever, cough, asthma, catarrh, toothache, diarrhea and rheumatism (Oyemitan *et al.*, 2006). The seeds are very important in the diets of women after childbirth, during which it is claimed that spices and herbs aid the contraction of the uterus (Aiyeloja and Bello, 2006). The fruit is used in entertaining guest; it is a sign of good reception in combination with cola nuts, garden egg and palm-wine. Also, it is used particularly during coronation, the new yam festival and marriage ceremonies in Igbo land (Enwere, 1998; Keay, 1989).

2.2 Pharmacological Effects of *D. tripetala*

2.2.1 Treatment of glaucoma

The seeds of *D. tripetala* have been shown to reduce intra ocular pressure (IOP) in humans. Timothy and Okeke (2008) reported that oral administration of 0.75 g of *D. tripetala* reduces the intraocular pressure for a short period, not lasting for 30 minutes after administration. The IOP was reduced by 17.30 % (12.90 mmHg) from the mean baseline of 15.60 mmHg (Timothy and Okeke, 2008).

2.2.2 Effect on bile secretion and biliary sodium and potassium ions

Oral administration of ethanolic extract of *D. tripetala* fruits at a dose of 85 mg/kg was shown to increase biliary sodium and potassium ions, while at a dose of 170 mg/kg the extract caused reduction of biliary sodium and chloride ions (Okafor, 1980). In a similar study, low dose (85 mg/kg) of extract reduced the rate of bile secretion while at a high dose (170 mg/kg) increase the rate of biliary secretion and bicarbonate ions concentrations without altering the saturation of cholesterol, conjugated and total bilirubin (Ofem *et al*., 2014).

2.2.3 Antinociceptive and anti-inflammatory effect

D. tripetala possesses significant antinociceptive and antiinflammatory activities (Oyemitan *et al*., 2006). Analgesic effects were shown to be mediated both centrally as well as peripherally while the antiinflammatory activity was shown to be effective in both early and late phases of inflammation` (Oyemitan *et al*., 2008).

2.2.4 Effect on oxidative stress and antioxidant activities

D. tripetala has been widely employed in the management of oxidative stress related diseases and infections (Aderogba *et al*., 2011). A 1, 1-diphenyl-2-picrylhydrazine (DPPH) guided fractionation was used to target and isolate the antioxidant constituents of the ethyl acetate solvent fraction of a 20% aqueous methanol leaf extract. Two flavonoid glycosides were isolated: avicularin and vitexin-2-O-rhamnoside (Aderogba *et al*., 2011). In a qualitative antioxidant assay, the compounds were shown to instantly bleach the DPPH purple colour indicating free radical scavenging potential antioxidant activity (Gyamfi *et al*., 1999). Unripe pepper fruit has higher antioxidant activities than the ripe fruit; this is due to the physiological changes that accompany ripening which increases total phenol and reduces vitamin C content hence reducing the antioxidant properties of the ripe fruit (Adedayo *et al*., 2010). In another study, the ability of the

ethanolic extract of the root of *D. tripetala* to inhibit lipid peroxidation in frozen heart muscle slices was investigated and the extract showed significantly ($p < 0.05$) higher antioxidant effect when compared with vitamin C suggesting that the root extract of *D. tripetala* is rich in antioxidants and can be used to enhance the preservation of meat during refrigeration (Okolie *et al*., 2014).

2.2.5 Antimicrobial and antihistaminic effect

D. tripetala is reported to have antimicrobial and antihistamic activities due to some phyto-components such as flavonoids, palmitic acid (hexadecaoic acid ethyl ester and n-hexadecanoic acid), unsaturated fatty acid, eicosanoic acid, ethyl ester and linoleic acid (Kumar *et al*., 2010). Ejechi *et al*., (1999) also reported the growth inhibition of tomato-rot fungi by phenolic acid and essential oil extract of *D. tripetala*.

2.2.6 Insecticidal effect

D. tripetala has protective ability on cowpea against storage insect pest. Anyaele and Amusan (2003) showed that hexane extract of *D. tripetala* has larvacidal activity against the mosquito larvae of *Aedes aegypti*. Similarly, the volatile compounds in *D. tripetala* have been reported by other workers to have larvacidal activities. The insecticidal activity of the plant powder was shown to increase with increased concentrations and days of exposure (Ukeh *et al*., 2012). Also, *D. tripetala* was shown to be effective in controlling the emergence of the adult and the destruction of the larval stages of the cowpea weevil (*Callosobruchus maculates*) and maize weevil (*Sitophillus zeamis*) completely (Okogu and Ekong, 1994). Asawalam *et al* (2007) showed that the bioactive component responsible for the insecticidal activity of *D. tripetala* to 2-nitroethyl-benzene and beta-phenyl nitroethane.

2.2.7 Hypnotic, anti-convulsant and anxiolytic effects

The oil of *D. tripetala* possesses hypnotic, anti-convulsant and anxiolytic effects when tested in mice. The essential oil and 1-nitro-2-phenylethane obtained from *D. tripetala* offered comparable dose-related protections against PTZ- and strychnine-induced convulsions. The compound 1-nitro-2-phenylethane exhibited dose dependent significant hypnotic, anticonvulsant and anxiolytic effects and it is the compound largely responsible for the neuropharmacological effects of the oil (Oyemitan *et al*., 2013).

2.2.8 Antihyperglycaemic effect of ethyl acetate extract of *D. tripetala*

The ethyl acetate extracts of *D. tripetala* enhanced glucose uptake in an *in vitro* study using 3T3-L1 adipocytes by partly mobilizing glucose uptake proteins from the interior of the cell to plasma membrane via the Golgi apparatus (Anaga and Asuzu, 2011). This mechanism may be responsible for the antihyperglycaemic effect of ethyl acetate extract of *D. tripetala* tested in diabetic rats (Anaga and Asuzu, 2010).

2.2.9 Other pharmacologic effects

D. tripetala is rich in α-linoleic acid, omega-3 fatty acid which has been reported to reduce the risk of cardiovascular diseases (William, 2000) and reducing hyperlipidemias thereby preventing coronary diseases (Kumar *et al*., 2010). Dietary α-linoleic acid in another study was shown to reduce the risk of prostate cancer (Koralek *et al*., 2006). Tannins, flavonoids and saponins found in the fruits are useful in the treatment of diabetes and rickets (Sparg *et al*., 2004; Reihemann *et al*., 1999). The phytochemical constituents in the extract of *D. tripetala* contribute to the medicinal activities of the plant.

D. tripetala fruits have been reported to contain important nutritive substances such as vitamins, minerals and fibre (Okwu and Morah, 2004). It was also indicated that the rich presence of essential oil (oleoresins) (Nwaogu *et al.*, 2007), phenolic acids and ethanol (Chandraseharen, 1994) determines its aromatic flavouring, coloring and pungent properties (Nwaogu *et al.*, 2007).

2.3 Phytochemical Constituents

Alkaloids, tannins, saponins, flavonoids, terpenoids, steroids and cardiac glycosides have been shown to be present in *D. tripetala* fruits (Adedayo *et al*., 2010). The major phytochemical components present in seeds of *D. tripetala* in a study by Elekwa *et al* (2011). are 9 –octadecanoic acid (30.53%), Linoleic acid ethyl ester (19.71%), Trichloroacetic acid (12.69%), octadecanoic acid ethyl ester (5.68%), 2-phenylethyl ester (2.38%), Caryophyllene (0.83%), 3-carene (0.72%), phenyethyl alcohol (0.53%), α-caryophyllene (0.36%), cyclopropane (0.29%), and α-cubebene (0.17%). These bioactive compounds are the basis for therapeutic potentials of medicinal plants.

2.3.1 Health benefits of dietry nutrients and elements obtained from *D. tripetala*

The total carbohydrate content of the fruit is high (62%). They comprise sugars such as glucose, sucrose and fructose, hemicellulose and pectin, which act as dietary fibre, add bulk to the diet and may, sometimes, act as a mild natural laxative (Enwere, 1998). *D. tripetala* fruit contains crude fibre (9.8%). Most ripe fruits contain no starch while some unripe fruits may contain a reasonable amount of starch. The fruits therefore are regarded as healthy foods and their consumption is beneficial in many ways. They provide dietary fibre, which among other things, promote bowel regularity, softens stool and enhances frequent waste elimination, such as bile acids, sterols and fat (Umoh, 1998). Fibre has a physiological effect on the gastrointestinal function of promoting the reduction of tracolonic pressure, which is beneficial in diverticular disease (Akobundu,

1999). Fibre also has a biological effect on the absorption and reabsorption of cholesterol and bile acid, respectively (Akobundu, 1999). It prevents the reabsorption of bile acids and, consequently, of dietary fat cholesterol. This, in turn, lowers cholesterol and prevents formation of plaques (Scala, 1974).

The fruits of *D. tripetala* contains high amount of potassium (2.48%) and calcium (1.80%). The sodium content is about 0.72%. Zinc, copper, manganese, cobalt, nickel and cadmium are available in trace quantities. (Okaka and Okaka, 2001).

Fruits of the plant also contains high (0.18%) amount of iron. Iron is known to be important in the human body because it is a component of haemoglobin. It helps in oxygen transport and, together with haemoglobin and ferrodixin, plays a vital role in body metabolism especially in humans (Okogun, 2002).

The fruit is also a good source of ascorbic acid, riboflavin, thiamine and niacin and can be used for the treatment of common cold and the control of other diseases such as prostate cancer (Okogun, 2002).

2.4 Toxicity Studies of *D. tripetala*

The effect of oral administration of ethanolic extract of *D. tripetala* fruits on haematological parameters in albino Wistar rats was investigated by Ikpi and Nku, (2008) and showed that *D. tripetala* extract, given at moderate to high doses may have haematotoxic effect. Anaga *et al* (2006), reported LD_{50} of the ethyl acetate root extract to be 1120 mg/kg when given intraoperitoneally with no remarkable change observed in the major organs at postmortem.

2.5 *Heligmosomoides bakeri*

H. bakeri (Figure 2.2) was first, described by Dujardin in 1845 (Durette-Desset, 1968) with four other nematodes from various rodents in France in a genus proposed as *Strongylus.* Hall in 1916 assigned the nematode to the genus *Heligmosomoides*. The name *Heligmosomoides polygyrus* was later used by Boulenger in 1922. Baylis in 1926 then named the parasite *Nematospyroides dubius* (Bryant, 1973). In 1968 the genus *Heligmosomoides* was re-established by Durette-Desset (Durette-Desset, 1968) who distinguished *Heligmosomoides* from *Heligmosomum* on the basis that only the latter had oblique cuticular ridges on the dorsal side. The worm is presently called *H. bakeri* (Behnke *et al*., 1991) to distinguish it from *H. polygyrus*, the dominant intestinal nematode of wood mice in Western Europe, which does not infect laboratory mice (*Mus musculus*) without the aid of an immunosuppressant. The life cycle is readily maintained in laboratory mice and adult worms are long lived (eight months 8) (Hewitson *et al*., 2011) so that frequent passage is not necessary. While nematodes such as *Nippostrongylus brasilensis* and *Trichinella spiralis* lasted only 2–3 weeks in the guts of their hosts when administered at typical relatively high dose rates (Day *et al*. 1979; Robinson *et al*. 1989). The infective larvae can be kept in aqueous suspension at 4°C for many months; a single culture can provide thousands of larvae for months of subsequent research. All these attributes have led to the parasite being adopted as a popular laboratory model of intestinal trichostrongylid infection (Behnke *et al*., 2009).



Figure 2.2 Photomicrograph of the adult worm of *H. bakeri* × 250 magnification (Wabo *et al*., 2011)

2.5.1 Life cycle of *H. bakeri*

The parasite has a direct life cycle. The eggs are passed out with the faeces of the host into the environment. After two days they hatch as larvae which are about 300 μm in length. The larvae moult 3 days after but retain the shed cuticle for protection. It is at this point that they become infective. The larvae shed the outer protective sheath after they are swallowed by a suitable host. The larvae then penetrate the submucosa of the duodenum where they further moulting twice seven days after the male and female adult worms emerge into the lumen of the duodenum and attach to the epithelial layer of the duodenum where they feed off the contents of the gut. The adult worms mate and the eggs that the female laid are shed in the faeces. The complete life cycle from egg to adult worm takes a minimum of 15 days, and the female worms will live inside their host for 8 months (Wabo *et al*., 2011). A schematic diagram showing the life cycle of *H. bakeri* is shown in figure 2.3.

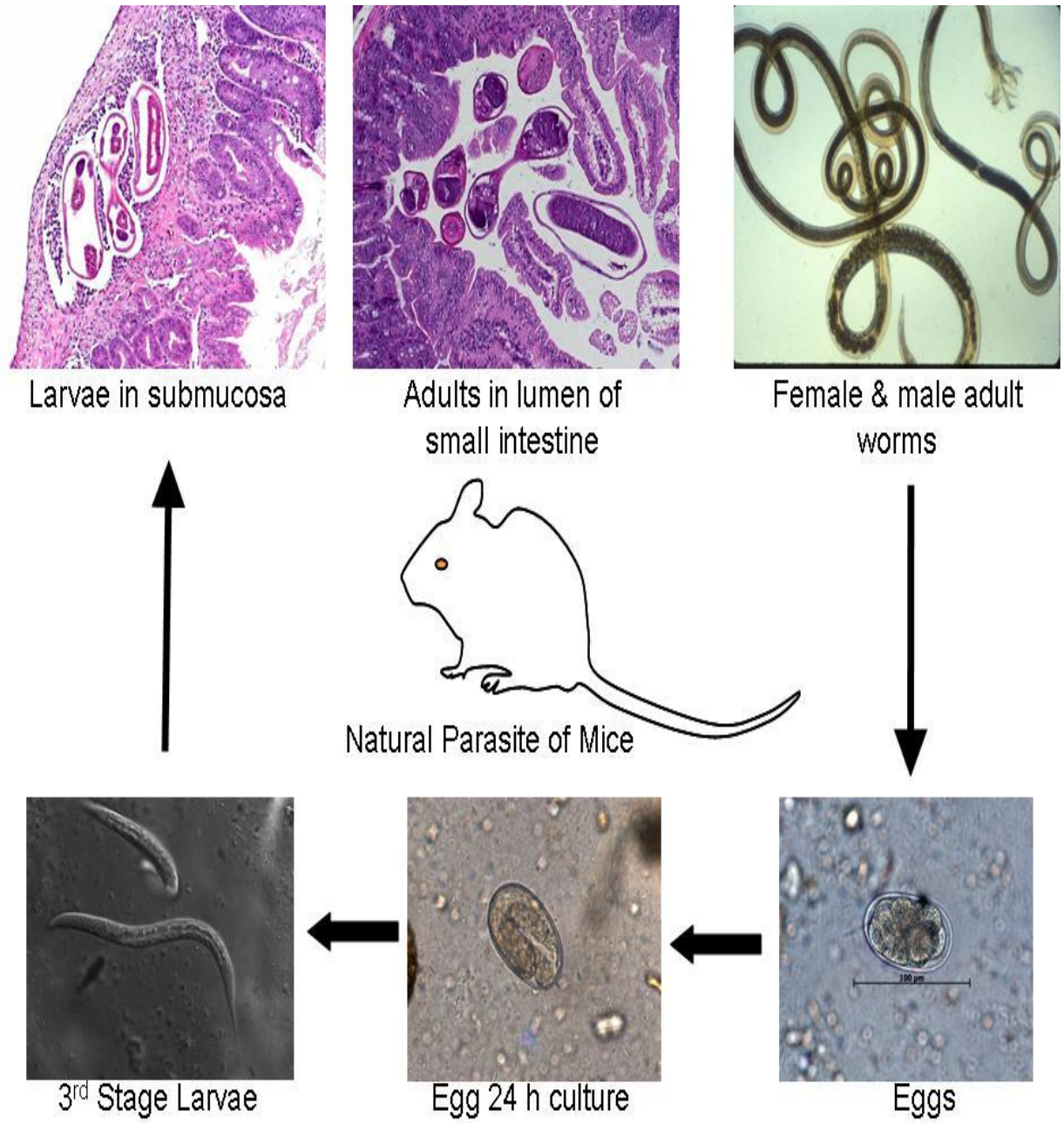


Figure 2.3 A Schematic diagram showing the life cycle of *H. bakeri* (Bryant, 1973)

2.6 Parasitic Biomarkers of Gastrointestinal Nematodes Infection

Knowledge of the epidemiology and pathophysiology of the gastrointestinal nematode has led to the identification of parasitic biomarkers for use as a diagnostic tool, for providing a threshold for anthelmintic treatment or for research.

2.6.1 Faecal Egg Count (FEC)

FEC is the most widely used parameter, both clinically and in studies on gastrointestinal nematode infections, because of their relative convenience and low cost. It relates to infective larvae ingestion (Eysker and Ploeger, 2000). FEC is viewed at a group-level rather than in individual animals. However, the counting sensitivity and proportion of homogenized eggs affects the validity of the data obtained (Denwood *et al*., 2012).

2.6.2 Pepsinogen

An increase in serum pepsinogen concentration reflects mucosal damage as a consequence of gastrointestinal nematode infection. There is hypoplasia and metaplasia of the parietal cells resulting in a decrease in acid production and a subsequent reduction of the pepsinogen transformation into pepsin. The accumulated pepsinogen escapes into the blood between the broken cell junctional complexes (Berghen *et al*., 1993). Some controversy still exists over the concentration of pepsinogen, which may be considered indicative of gastrointestinal nematode infection Pepsinogen assay methods are mainly based on the immediate conversion of the proenzyme into the proteolytic enzyme in acid conditions. (Charlier *et al*., 2011)

2.6.3 Antibodies

In contrast to pepsinogen determination, a commercial assay for quantifying antibodies to gastrointestinal nematodes is currently available (Svanovir®O. ostertagi-Ab, Uppsala, Sweden). Antibody concentration against gastrointestinal nematodes correlate

reasonably well with pepsinogen concentration and have been proposed as an alternative for monitoring purposes (Berghen *et al*., 1993; Eysker and Ploeger, 2000). An enzyme-linked immunoabsorbent assay (ELISA) using a crude adult *Ostertagia ostertagi* antigen has been available (Keus *et al*., 1981). Using antibody measurement instead of pepsinogen for monitoring gastrointestinal nematode infections has proven to be more reliable. However, the Svanovir® assay has been primarily designed for use in adult cows using milk samples (Charlier *et al*., 2009) and an extensive evaluation for use in calves has not yet been performed. The pepsinogen concentration provides a more direct assessment of the clinical effects of gastrointestinal worm infection (abomasal damage) than specific antibodies as a positive antibody response does not indicate either clinical disease or functional immunity; it simply indicates prior or current exposure.

2.6.4 Pasture Larval Count

Pasture larval counts are laborious and are rarely used as routine methods of assessment of risk of nematode infections. However, they are particularly useful in longitudinal studies on the population dynamics of nematodes (Eysker and Ploeger, 2000)

2.6.5 Gastrin

Gastrin is a hormone produced by the G cells present mainly in the glands of the pyloric region of the abomasum. The release of gastrin is mediated by vagal stimulation, mechanically by stomach distension and chemically by peptides, amino acids or calcium. Through a negative feedback mechanism its release can be triggered by high pH levels as happens in some gastrointestinal nematode infection like ostertagiosis (Purewal *et al*., 1997).

The use of gastrin as a diagnostic marker has been examined in both sheep and cattle. Measurement of gastrin concentration is expensive and yields no more useful further information than the cheaper pepsinogen concentration assay (Entrocasso *et al*., 1986).

2.7 Anthelmintic Screening of Plants

Antihelmintic screening of medicinal plants, involves setting up a suitable nematode *in vitro* model that can evaluate potential product and preparation of extracts that can be redissolved in solvents that are miscible in the test medium and are at concentrations well tolerated by the nematode system used for screening (Katiki *et al*., 2011).

2.7.1 *In vitro* anthelmintic assay

Anthelmintic screening of medicinal plants reported in literatures are mostly *in vitro* studies using some helminth model such as *Ascaridia galli* (Abdelquader *et al*., 2012), *Ascaris lumbricoides* (Mali and Mehta, 2007), *Haemonchus contortus* (Alawa *et al*., 2003; Ademola and Eloff, 2011), trichostrongylid; *Heligmosomoides bakeri* (Wabo *et al*., 2011; Payne *et al*., 2013), *Trichostrongylus colubriformis* (Bizimenyenra *et al*., 2006), *Oesophagostomum* and *Cooperia* (Chiezey *et al*., 2000), Indian adult earthworm; *Pheretima posthuma* (Manoj and Rajesh, 2011). A recent model developed for *in vitro* anthelmintic studies is the use of house fly worm (*Stomoxys calcitrans*). This new model is quite easy, prominent, eco-friendly, and the method is reproducible in all aspects such as equal age, size, and weight of worms used for the experiment (Murugamani *et al*., 2012).

In vitro studies provide useful information and scientific basis for further *in vivo* studies (Mali and Mehta, 2007). Parameters often measured during *in vitro* anthelmintic studies are shown in Table 2.1.

Table 2.1. *In vitro* bioassay methods of gastro-intestinal helminth parasite

BIOASSAY	**TARGET STAGE(S)**	**PROCESS DISRUPTED**
Egg hatch test	Eggs	Hatching of eggs to L_1
Larval migration inhibition test	L_3	Locomotion of L_3
Larval feeding inhibition test	L_1	Disruption of feeding of L_1
Larval exsheathment assay	L_3	Exsheathment of L_3
Larval development assay	Eggs, L_1, L_2, L_3	Disruption of development to L_3
Adult motility test	L_5/Adult worm	Motility (Viability) of adult worms

Jackson and Gordon (2013)

2.7.2 *In vivo a*nthelmintic activity

In vivo studies of anthelmintic activity of plant extracts have been conducted using parasites such as *Nippostrongylus brasiliensis* in rats (Suleiman *et al*., 2005; Jegede *et al*., 2009), *Ancylostoma caninum* in dogs (Adedapo *et al*., 2007), *Haemonchus contortus* in sheep and goat (Alawa *et al*., 2003; Hussain *et al*., 2008, 2011), *Heligmosomoides bakeri* in mice (Ngongeh and Fakae, 2011), *Ascaris suum* in pigs (Satrija *et al*., 1994), *Ascaridia galli* in chicken (Abdelqader *et al*., 2012). The parameters for such activity included expulsion of worms from their hosts or reduction in the number of eggs per gram of faeces (EPG) passed by the infected hosts compared to commercial anthelmintic treated animals (Jegede *et al*., 2009). Others also measured the weight and the packed cell volume (PCV) of the animals (Githiori, 2004; Ngongeh and Fakae, 2011).

2.8 Control of Helminth Infection

Several methods are employed for the control of helminth infection. Some of these methods include: Chemical control such as use of anthelmintics, use of repellant and pheromones; and non-chemical control such as biological control, worm vaccines, selection for host resistance, grazing management, nutritional management, interspecific competition and male sterile technique.

2.8.1 The use of anthelmintics

The use of anthelmintics by livestock producers has been beneficial in the control of helminthosis (Abdu and Faya, 2000). Anthelmintics are drugs or chemical agents that reduce parasite burdens in the animals to a tolerable level; they kill the parasites (vermicide), inhibit their growth or paralyse them (vermifuge). They also reduce the build-up of infective worm larvae on the pasture or eggs in the environment (Aliu,

2006). The major classes of synthetic drugs used in control of helminth parasites are categorised on the basis of chemical structure or mode of action (Jones, 2013).

2.8.1.1. Piperazine

Piperazine was first used as an anthelmintic in the 1950s. It acts as a weak GABA-mimetic and causes a flaccid, reversible paralysis of body wall muscle. Single channel recordings provide evidence that it is a low efficacy, partial agonist at GABA-gated chloride channels (Martin, 1985).

2.8.1.2 Benzimidazoles

The first of this class, thiabendazole, was discovered in 1961 (Borgers and De Nollin, 1975) and subsequently a number of further benzamidazoles such as mebendazole, flubendazole, fenbendazole, oxfendazole, oxibendazole, albendazole, albendazole sulfoxide, thiophanate, febantel, netobimin and triclabendazole were introduced as broad spectrum anthelmintics (McKeller and Jackson, 2004). They are most widely used in veterinary medicine to treat nematode infections (Campbell, 1990; Lanusse and Prichard, 1993) due to their wide margine of safety and efficacy. Its anthelmintic efficacy is due to their ability to compromise the cytoskeleton through a selective interaction with β-tubulin (Borgers and De Nollin, 1975). The effects of benzimidazoles on nematodes include impaired locomotion, reproduction and a detrimental effect on oocytes. The benzimidazoles are effective against roundworm, whipworms and hookworms in most species. In horses, they are effective against strongyles and pinworms (Campbell, 1990), in dogs and cats fenbendazole are effective against taenia (Richard and Arundel, 1985; McKellar *et al*., 1990). Mebendazole, thiabendazole and fenbendazole are highly effective against *Haemonchus contortus* and *Trichostrongylus columbriformis* in sheep (Kelly *et al*., 1977).

2.8.1.3 Imidazothiazole (*levamisole*) *and Tetrahydropyrimidines* (*pyrantel and morantel*)

These anthelmintics are nicotinic receptor agonists (Aceves *et al*., 1970) and elicit spastic muscle paralysis due to prolonged activation of the excitatory nicotinic acetylcholine (nACh) receptors on body wall muscle.

2.8.1.4 Praziquantel

Praziquantel was discovered in 1975 (Harder, 2002) and has been in use for the treatment of schistosomes and a wide range of cestode infections (Seubert *et al*., 1977). It acts by increasing calcium influx across the parasite membrane and cause rapid muscle contraction of the parasite leading to paralysis and expulsion (Martin, 1997).

2.8.1.5 Proton ionophores

Proton ionophores are salicylanilide derivatives (Cringoli *et al*., 2006). They were originally developed as fungicides and general antimicrobials but they were later shown to possess potent anthelmintic activity (Iglesias *et al*., 2002). Members of this group include niclosamide, tribromsalane, clioxanide, closantel and rafoxanide (Swan, 1999). Closantel and rafoxanide are important anthelmintic used in treating *Haemonchus contortus* and *Fasciola* spp infection in sheep and cattle (Van Wyk *et al*., 1997). Niclosamide is widely used for the treatment and control of cestode infections in several animal species (Lateef *et al*., 2008). They act by selectively uncoupling oxidative phosporylation in the parasite mitochondria (Martin, 1997).

2.8.1.6 Paraherquamide

Paraherquamide A and marcfortine A are both members of the oxindole alkaloid family, originally isolated from *Penicillium paraherquei* and *Penicillium roqueforti*,

respectively (Zinser *et al*., 2002). Paraherquamide and its derivative 2-deoxy-paraherquamide induce flaccid paralysis in parasitic nematodes. Paraherquamide also blocks the actions of other nicotinic agonists, but not equipotently (Zinser *et al*., 2002; Robertson *et al*., 2002).

2.8.1.7 Macrocyclic lactones or Ivermectin (*macrocylic lactones and milbemycins*)

Ivermectin was introduced as an anthelmintic in the 1980s by Merck. It is a semi-synthetic derivative of avermectin which is a large macrocyclic lactone fermentation product of the micro-organism *Streptomyces avermitilis*. It is remarkably potent and persistent in its effect. Ivermectin analogues include moxidectin, milbemycin oxime, doramectin, selamectin, abamectin and eprinomectin (Haber *et al*., 1991). Ivermectin elicits a potent and persistent paralysis of nematode pharyngeal (Pemberton *et al*., 2001) and body wall musculature (Kass *et al*., 1982). It has been shown to interact with a range of ligand-gated ion channels including α7nACh receptors (Krause *et al*., 1998), acetylcholine-gated chloride channels (Bokisch and Walker, 1986), GABA-gated chloride channels (Holden-Dye and Walker, 1990), histamine-gated chloride channels (Zheng *et al*., 2002), glycine receptors (Shan *et al*., 2001) and P2X4 receptors (Khakh *et al*., 1999). However, it has high affinity for nematode glutamate-gated chloride channels (GluCl) that correlates with its potent anthelmintic activity.

2.8.1.8 Emodepside (*cyclodepsipeptides*)

The cyclodepsipeptide molecule, emodepside, is a semi-synthetic derivative of PF1022A, a fermentation product obtained from the fungus, *Mycelia sterilia* (Harder and von Samson-Himmelstjerna, 2002). It is effective against isolates of parasites that are resistant to benzimidazole, levamisole and ivermectin (Saeger *et al*., 2001). It has a

homology to mammalian latrophilins, a class of G protein-coupled receptors which bind the neurotoxin, latrotoxin. Latrotoxin paralyses mammals by triggering neurotransmitter release, and thus the identification of latrophilin as an emodepside receptor raised the intriguing possibility that emodepside may cause paralysis of nematodes by stimulating excessive neurotransmitter release at neuromuscular sites (Bull *et al*., 2007). The effects include slowed development, inhibition of pharyngeal pumping, decreased locomotion (forward movement is most affected at low concentrations), and inhibition of egg-laying.

2.8.1.9 Nitazoxanide

Nitazoxanide, a pyruvate ferredoxin oxidoreductase inhibitor, acts against a broad spectrum of protozoa and helminths that occur in the intestinal tract. It is currently used for the treatment of protozoal infections.The site of action of this compound may be anaerobic electron transport enzymes (Gilles and Hoffman, 2002). Nitazoxanide has been examined on growth and development of *nematodes* (Fonseca-Salamanca *et al*., 2003). Therefore the efficacy of this compound is relatively low compared to other anthelmintic agents.

2.8.1.10 Other forms of chemical control methods

The use of poisons, repellants and pheromones to control helminths has also been practiced in livestock with varying results and levels of efficacy (De and Sanyal, 2009; Sargison, 2011).

2.8.2 Non-Chemical Control methods

2.8.2.1 Grazing management

Apart from the use of drugs, there are husbandry practices and measures that are used to control helminth parasites. Avoiding early morning grazing by farmers until the sun has risen to help prevent the ingestion of infective larvae of helminth parasites from the pasture. Rapid rotational grazing system, tethering and zero grazing (Semenye *et al*., 1992) of the animals are carried out to maximize pasture consumption and encourage immunity of the animal as well as maximizing productivity (Bukhari and Sanyal, 2011).

2.8.2.2 Biological control

Biological control can be natural or applied. Certain organisms like some fungi are known to attack and destroy helminths for example *Arthobotrys oligospora* and *Monacrosporium spp*. have been tested as biological control agents (De and Sanyal, 2009). With regard to the effect of nematophagous fungi against cattle parasitic nematodes, it is possible to reduce the number of infective larvae on herbage and subsequently to reduce the number of nematodes establishing in the grazing animals (Wolstrup *et al.* 1994; Nansen *et al.* 1995). A nematode-killing fungus, *Duddingtonia flagrans*, discovered in New Zealand (Skipp *et al.* 2002), showed a trapping efficiency rate of 78% and activity for up to 90 days on pasture, providing a viable alternative to reduce animal mortality from nematode infections (Waghorn *et al.* 2003). In South Africa, local strains of *D. flagrans* have been identified (Durand *et al.* 2005).

2.8.2.3 Nutrition

Well-nourished animals are generally more resistant to the effects of parasite infection. Therefore, nutritional supplementation may reduce the requirement for anthelmintic

control (Knox *et al*., 2006; Torres-Acosta *et al.* 2004). Additional dietary protein (Torres-Acosta *et al.* 2004), selenium (Au Yeung *et al.* 2005; Smith *et al.* 2005), as well as minerals (Islam *et al.* 2006; Koski and Scott, 2003) may each play a role in countering infections presumably through mechanisms such as enhancing host immunity or maintaining digestive tract integrity.

2.8.2.4 Host resistance

Increasingly attractive alternatives for the control of gastrointestinal nematodes are the identification of the host genes that influence the acquired or innate resistance to the parasites and the use of the vast potential of the host genome to reduce parasite transmission (Gasbarre *et al.* 2001). Studies in the USA demonstrated that the number of nematode eggs per gram (EPG) in faeces of pastured cattle is strongly influenced by host genetics (Leighton *et al*., 1989) and that the heritability of this trait is approximately 30% (Gasbarre *et al*., 2001). This suggests that genetic management of a small percentage of the herd could considerably reduce overall parasite transmission (Gasbarre *et al.*, 2001). Hence, according to Gasbarre *et al* (2001), accurate identification of the genes that control resistance will offer the producer several alternatives for disease control. The small percentage of susceptible animals can be targeted for drug administration and this approach would reduce both the cost of the anthelmintics that are used and the odds for selection of drug-resistant mutants.

2.8.2.5 Vaccination

Vaccinations would prevent rather than cure infections and represent a more sustainable and environmentally friendly form of parasitic control compared to drugs. However, with the exception of the vaccine against *D. viviparus*, no vaccines are currently

available commercially for the control of helminth infections in ruminants (Knox and Smith, 2001). Few of the experimental vaccines have proceeded to or successfully passed the next phase of development, which is the large-scale, cost-effective production of the vaccine (Geldhof *et al.*, 2007). However, Dairy calves vaccinated three times with a gut membrane from *H. contortus* immunogen and then challenged with *H. contortus* and *H. placei* showed a significant reduction of egg counts for *H. contortus* (100%) and reduction in worm count for *H. placei* (68.4%) (Bassetto *et al*., 2010).

2.8.2.6 Feeding anthelmintic plants or crops

Plants or crops with natural anti-parasitic properties play a role in grazing systems for livestock (Waller and Thamsborg, 2004). Several tannin-containing forages, in particular those with condensed tannins, showed anthelmintic activity against gastrointestinal nematodes of sheep and goats (Hoste *et al.*, 2006). Extracts of three tannin-containing plants, *Onobrychis viciifolia*, *Lotus pedunculatus* and *Lotus corniculatus*, were shown to have *in vitro* anthelmintic activity against the cattle nematodes *O. ostertagi* and *C. oncophora*, with tannins from *L. pedunculatus* being more active than those of *O. viciifolia* and *L. corniculatus* (Novobilsky *et al*., 2011).

Table 2.2 Plants that were shown to have Anthelmintic Activity.

Plant species	Parts used/ Extract type	Target parasite	Method of evaluation	Reference
Anarcadium occidentale	Acetone extract of leaves	*Haemonchus contortus* eggs and larvae	*in vitro*	Ademola and Eloff, 2011
Annona sanegalensis	Water extract of stem bark	*Haemonchus contortus* eggs and larvae	*in vitro*	Alawa *et al*., 2003
Occimum gratissimum	Leaves essential oil	*Haemonchus contortus* eggs and larvae	*in vitro*	Pessoa *et al*., 2002
Artemisia brevifolia	Crude methanol extract of whole plant	*Haemonchus contortus* eggs, larvae and adult	*in vitro* and *in vivo*	Iqbal *et al*., 2004
Mucuna pruriens	Leaves	*Haemonchus contortus* adult	*in vivo* in lamb	Huisdena *et al*., 2010
Azadiracha indica	Ethanol extract of powdered seed	*Haemonchus contortus* eggs and larvae	*in vitro*	Hordegen *et al*., 2006
Musa paradisiacal	Crude methanol extract of leaves	*Haemonchus contortus* eggs, larvae and adult	*in vitro* and *in vivo*	Hussain *et al*., 2011
Peltrophorum africanum	Acetone extract of leaves, root and stem bark	*Trichostrongylus colubriformis*	*in vitro*	Bizimenyenra *et al*., 2006
Zingiber officinale	Methanol extracts of Rhizomes	*Haemonchus contortus* adult	*in vitro*	Iqbal *et al*., 2001

Table 2.2 continued

Plant species	Parts used/ Extract type	Target parasite	Method of evaluation	Reference
Allium sativum	Methanol extract of bulb	*Haemonchus contortus* eggs and larvae	*in vitro*	Iqbal *et al*., 2001
Moringa olifera	Ethanol extract of leaves and stem bark	*Ascaris suum* adult	*in vitro*	Wasswa and Olila, 2006
Carica papaya	Ethanol extract of leaves	*Ascaris suum* adult	*in vitro*	Wasswa and Olila, 2006
Daniellia oliveri	Stem bark	*Haemonchus contortus* eggs and larvae	*in vitro*	Adama *et al*., 2009
Picrolemma spruce	Water and ethanol extract of stem bark and root	*Haemonchus contortus* eggs and larvae	*in vitro*	Nunomura *et al*., 2006
Rumex abyssinicus	Alcohol extract of leaves	*Haemonchus contortus* eggs and larvae	*in vitro*	Eguale *et al*., 2011
Aframomum danielli	Water and ethanol extract of seed	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Kollins *et al*., 2012
Embelia rowlandii	Cold and hot water and ethanol extract of seed	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Payne *et al*., 2013
Dichrocephala integrifolia	Aqueous and ethanol extract of leave	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Wabo *et al*., 2013

Table 2.2 continued

Plant species	Parts used/ Extract type	Target parasite	Method of evaluation	Reference
Schinopsis spp (Quebracho tannin)	Extract of the bark dissolved in water	*Heligmosomoides bakeri* eggs and larvae	*in vivo* in mice	Ngongeh and Fakae., 2011
Chenopodium ambrosoides	Hexane and ethanol extract of leaves	*Heligmosomoides bakeri* eggs and larvae	*in vitro*	Wabo *et al*., 2011
Spigelia anthelmia	Crude ethanol, chloroform and ethylacetate extract of plant	*Nippostrongylus braziliensis*	*in vivo* in rats	Jegede *et al*., 2009
Afzelia Africana	Crude methanol, chloroform and n-butanol extract of stem bark	*Nippostrongylus braziliensis*	*in vivo* in rats	Simon *et al*., 2012
Saraka indica	Ethanol extract of leaves	*Pheritima posthuma (*indian earthworm)	*in vitro*	Sarojini *et al*., 2011
Corallocarpus epigaeus	Alcohol and water extract of root	*Pheritima posthuma*	*in vitro*	Kirubha *et al*., 2011
Xylopia aethiopica	Crude methanol extract of seeds	*Nippostrongylus braziliensis*	*in vivo* in rats	Suleiman *et al*., 2005
Persicaria hydropiper	Methanol extract of the aerial part	*Pheritima posthuma*	*in vitro*	Raihan *et al*., 2012
Cassia auriculata	Acetone and ethanol extract of the plant	*Eicinia fetida* (Earthworm)	*in vitro*	Gaikwad *et al*., 2011

Table 2.2 continued

Plant species	Parts used/ Extract type	Target parasite	Method of evaluation	Reference
Cymbopogon ciratus	Methanol and aqueous extract of leaves	*Pheritima posthuma*	*in vitro*	Tare *et al*., 2011
Combretum molle	Acetone extract of leaves	*Haemonchus contortus* eggs and larvae	*in vitro*	Ademola and Ellof, 2010

CHAPTER THREE

MATERIALS AND METHODS

3.1 Location of the Experiment

The *in vitro* and *in vivo* experiments were conducted in the Departments of Veterinary Pharmacology and Toxicology, Veterinary Parasitology and Entomology, Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria.

3.2 List of Materials Used

3.2.1 Equipment and apparatus

Ninety six-well microtitre plates, light microscope, glass slides, cover slips, Pasteur pipettes, petri dishes, filter papers, sample bottles, masking tape, permanent makers, digital weighing balance, beakers, measuring cylinders, stirring rod, funnels, test tubes (centrifuge tubes and flotation tubes), egg counter, McMaster slides, syringes of different sizes, conical flask, separatory funnels, cotton wool, gauze, water bath, retort stand, glass bottles, scissors and mice cages.

3.2.2 Reagents, drugs and chemicals

Methanol, hexane, ethylacetate, butanol (JHD Guangdong Guanghua Sci-Tech Co., Ltd. Shantou, Guangdong, China). Distilled water, normal saline (0.9 % sodium chloride), albendazole (Tuyil Pharmaceutical Ltd., Nigeria), chloroform, tween 80, flotation media (saturated salt solution) and 10% formalin.

3.3 Plants Collection, Identification and Preparation

D. tripetala fruits were purchased from Abubakar Gumi Central Market, Kaduna. Sample of the fruits was sent to the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria for identification. A voucher number of 2737 was issued and the sample of the fruits was kept for future reference in the Herbarium. The fruits were washed and dried under shade to prevent destruction by sun rays and heat. The dried fruits were made into powder form using wooden mortar and pestle.

3.3.1 Plants extraction

One kilogram of the powdered fruits was exhaustively extracted in a soxhlet apparatus using absolute methanol as solvent. The powdered plant and methanol were mixed in the ratio of 1:3. The liquid extract obtained was concentrated *in vacuo* at 40 ^{o}C using rotatory evapourator. The dried extract was kept at 4^{o} C in the fridge at the Toxicology laboratory of the Department of Veterinary Pharmacology and Toxicology until required.

3.3.2 Solvent partitioning of extracts

The methanol extract was partitioned using solvents of different polarities by methods described by Handa *et al* (2008). Fifty grams of the solid crude methanol extract was dissolved in 500 mls of distilled water (ratio of 1:10 w/v) to form an aqueous methanol extract (AME) and serially partitioned with hexane, ethylacetate and butanol as solvents using a separating funnel. Each partition process was repeated twice using equal volume (500 mls) of each solvent, and similar fractions were pooled together and concentrated *in vacuo* (Handa *et al.*, 2008).

3.4 Phytochemical Screening of the Extracts

The crude methanol extract (CME), aqueous methanol extract (AME), hexane extract (HE), ethylacetate extract (EAE), and butanol extract (BT) were subjected to phytochemical screening using methods described by (Evans, 2007). One gram of each portion of the extract was dissolved in 100 ml of distilled water and the following phytochemical tests were conducted:

3.4.1 Test for carbohydrates

3.4.1.1 Molisch's test

Few drops of Molisch's reagent were added to 3 ml of the dissolved extract in a test tube followed by the addition of 2 ml of concentrated sulphuric acid (99.9%) which was carefully poured down the side of the test tube. A purple to reddish ring at the interface was indicative of the presence of carbohydrate.

3.4.1.2 Fehling's test

Five mililitres of each portion of the extract was poured into 2 seperate test tubes and 5 ml of the mixture of Fehling's solution A and B in the ratio of 1:1 were added. The mixture was boiled on a water bath for 2 minutes. The appearance of a brick-red precipitation is indicative of the presence of glycone due to hydrolysis of glycosides or reducing sugar.

3.4.2 Test for free anthraquinones (Modified Borntrager's test)

One gram of the extract was mixed with 5 mls of 10% hydrochloric acid for 3minutes. It was then boiled and filtered, the filtrate cooled and extracted with 5mls of 99.9 % benzene. The benzene portion was pipetted off and shaken gently in a test tube with 2 mls of 10% ammonium hydroxide. The production of pink-red colouration is indicative of free anthraquinones.

3.4.3 Test for cardiac glycosides

3.4.3.1 Kella-Killiani Test

Half a gram of the powdered extract was dissolved in 2 ml glacial acetic acid in a test tube. Thereafter the test tube was held at an angle of 45 degrees while carefully adding 1ml of 99.9 % sulphuric acid down the side using a pipette. The appearance of a purple ring colour at the interface indicated the presence of cardiac glycosides.

3.4.4 Test for saponins (frothing test)

Half a gram of the powdered extract was dissolved in 10 ml of distilled water. The mixture was vigorously shaken for 30 seconds and allowed to stand for 30 minutes. The formation of a honey-comb froth that persists for more than 30 minutes is indicative of the presence of saponins.

3.4.5 Test for steroids triterpenes (Lieberman-Burchards test)

One mililitre of 99.9 % acetic anhydride was added to 1 ml of the extract and 1 ml of 99.9 % sulphuric acid was added down the side of the test tube. Red, pink or purple colour observed immediately after the addition of the acid indicated the presence of triterpenes while blue or blue-green colouration observed much later indicated the presence of steroids.

3.4.6 Test for flavonoids

3.4.6.1 Sodium hydroxide test

Half a gram of the powdered extract was dissolved in 5 ml of water in a test tube. Thereafter, three drops of 5% sodium hydroxide were then added to the solution. The appearance of yellow colouration indicated the presence of flavonoids.

3.4.6.2 Shinoda Test

Half a gram of the powdered extract was dissolved in 2 ml of 50 % methanol and heated. This was followed by the addition of metallic magnesium chips and four drops of 99.9 % hydrochloric acid. A red or orange colour is indicative of the presence of flavonoids.

3.4.7 Test for tannins (ferric chloride test)

One gram of each portion of the extract was dissolved in 10 ml of distilled water and the solution was filtered. Two drops of ferric chloride solution were added to the filtrate. The presence of a blue-black precipitate indicated the presence of hydrolysable tannins, while a green precipitate indicated the presence of condensed tannins.

3.4.8 Test for alkaloids

3.4.8.1 Meyer's test

Few drops of Meyer's reagent were added to 5 ml of the extract already dissolved in distilled water. The appearance of a cream precipitate indicated the presence of alkaloids.

3.4.8.2 Dragendoff's test

Half a gram of the powdered extract was dissolved in 10 ml of distilled water and 3 drops of Dragendoff's reagent were added to the solution. Redish brown precipitate indicated the presence of alkaloids.

3.4.8.3 Wagner's test

One gram of each portion of the extract was dissolved in 10 ml of distilled water in a test tube and 2 drops of Wagner's reagent were added to the solution. The presence of a whitish precipitate indicated the presence of alkaloids.

3.5 Experimental Animals

Swiss albino mice of both sexes were obtained from the Animal House, Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria. Eighty five apparently healthy albino mice (*Mus musculus*) of both sexes weighing between 18 and 20 grams were used for the experiment. The animals were preconditioned to the laboratory environment in mice cages at room temperature for two weeks. During this period, those intended for anthelmintic studies were screened for natural helminth infections and those found to habour gastrointestinal helminthes were dewormed using albendazole (10 mg/kg) orally. All the mice were maintained on commercial grower poultry feed with a nutrient composition of 13% crude protein, 15% crude fibre, 8% fat and 1.25% minerals. Water was provided *ad libitum.* Wood shavings were used as bedding and were changed every two days. The mice were grouped and kept in cages. Each group consisted of five mice which were identified by marks on their tails using permanent markers of different colours.

3.6 Acute Toxicity Studies

The method described by Lorke (1983) was used to determine the median lethal dose (LD_{50}) of the crude methanol extract in mice. In the first phase, mice were divided at random into three groups consisting of 3 mice each. Groups 1, 2 and 3 were administered with the extract at 10, 100, 1000 mg/kg, respectively per os. The mice were observed over a period of 48 hours for signs of toxicity and mortality. In the second phase of the trial, another set of mice were divided into four groups of one mouse each. Groups 1, 2 and 3 were treated orally with 1600, 2900 and 5000 mg/kg of the extract, respectively. Group 4 was given distilled water at 5 ml/kg maximum volume administrable to rats/mice at a time using the oral route (Ibrahim *et al.,* 1983). The animals were observed for 24 hours for signs of toxicity and mortality.

3.7 Post-Mortem and Histopathological Studies

At the end of the LD_{50} determination, all mice administered with the extract and those treated with distilled water were euthanized and the internal organs were grossly examined for pathological changes. The livers, kidneys, lungs and spleen were removed, fixed in bouin's solution and processed for histopathological examination. The slides were stained with haematoxylin and eosin stains and examined under a light microscope at ×500 magnification for any histhological changes (Luna, 1960).

3.8 Egg Culture and Larval Recovery

3.8.1 Infection of worm free mice

The third stage (infective) larvae of *H. bakeri* were obtained from the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria and were used to infect worm free mice. Each mouse was given 200

L_3 contained in 0.2 ml of water orally using a blunted tip 18 G needle mounted on a 1ml syringe.

Two weeks post-infection, droppings from the infected mice were obtained by placing each mouse on a clean plastic cage. The faeces produced by each mouse were collected into a labeled container and confirmed for the presence of *H. bakeri* eggs using the simple floatation method (Soulsby, 1982).

3.8.2 Recovery of *H. bakeri* eggs

The method of Micheal *et al* (2001) was used to obtained fresh eggs of *H. bakeri*. Three grams of faeces from previously infected mice were collected in a centrifuge tube and homogenized using glass rod in 12 ml of saturated sodium chloride solution. The solution was filtered through a tea sieve and the filtrate centrifuged at 5000 g for 5 minutes. The supernatant was poured into a beaker and 100 mls of distilled water were added to it. The mixture was further centrifuged at 5000 *g* for 5 minutes. Using a 20 ml syringe, the supernatant aspirated and discarded and more water was added to the sediment and re-centrifuged further for 5 minutes at 5000 *g*. The sediment obtained after aspirating the supernatant with a syringe was spread on a glass slide and examined under a light microscope at ×10 magnification for the presence of *H. bakeri* eggs (Appendix I). The recovered eggs were used for the *in vitro* test (Wabo *et al*., 2010; Kollins *et al*., 2012; Payne *et al*., 2013).

3.8.3 Culture of eggs

Eggs were cultured into infective L_3 stage according to the method described by Chiejina and Fakae (1984). Four grams of faecal sample was collected from infected mice and homogenized in 50 ml centrifuge tube using distilled water. The fecal slurry was filtered using a tea sieve. The filtrate was centrifuged at 5000 *g* for 5 minutes and

the supernatant aspirated using 20 ml syringe. The sediment was poured into petri dishes having their bottom covered with Whatman`s filter paper. The petri dishes were covered, left at room temperature and moistened daily with distilled water for 7 days to obtain the L_3 stage.

3.8.4 Harvesting and preservation of infective *H. bakeri* larvae

The filter paper on the 7-day old culture was lifted with a forcep and sprayed with distilled water to wash the larvae into a clean petri dish. Thereafter, the filter paper was removed and discarded. Safely using a Pasteur pipette, 2 drops of the water in the petri dish containing the larvae were placed on a glass slide and examined under the light microscope for the presence of motile L_3 (Appendix II).

3.8.5 Determination of larval count for artificial infection and larvicidal test

The method described by Suleiman (2002) was adopted. The larval suspension was placed in a beaker and stirred. Using a 1 ml syringe, 0.1 ml of the solution was aspirated and stripped on a glass slide followed by the addition of a drop of iodine to immobilize the larvae so as to make counting easier and the slide examined under a light microscope at ×10 magnification. The process was repeated five times and the average number of the larva counted was recorded. A concentration and dilution technique was used and the volume adjusted such that about 200 larvae were in 0.2 ml of the solution.

3.9 *In vitro* Anthelmintic Screening of *D. tripetala* Fruit Extract and Fractions

3.9.1 Evaluation of ovicidal activity of the extracts (Egg hatch inhibition test)

The *in vitro* ovicidal effect of the extracts was done according to methods described by Coles *et al* (1992) and Wabo *et al*. (2010). Two hundred microlitres of egg suspension containing about 200 fresh eggs was aspirated using a pipette and put into each well of a labeled 96-well microtitre plate. In addition, 200 µl of different concentration (2.5, 1.25,

0.6, 0.3, 0.15 and 0.07 mg/ml) of each fraction was tested for ovicidal activity. Similarly, albendazole at the same concentration and distilled water were used as treated and untreated controls, respectively. Each experimental set up was done in triplicate. The plates were covered with foil papers to prevent evaporation and then left to stand at room temperature for 48 hours. Thereafter, using a pipette, the content of each well of the micro titre plate was placed on a seperate glass slide using a pipette and examined microscopically at ×10 magnifications. All the unhatched eggs as well as the l_1 in each well were counted and recorded.

The percentage inhibition of egg hatching by the test substances was calculated using the formula:

$$\text{Percentage Egg Inhibition} = \frac{P_{control} - P_{test}}{P_{control}} \times 100$$

Where P_{test} = mean number of eggs that hatched in the treated wells

$P_{control}$ = mean number of eggs that hatch in the untreated control well.

3.9.2 Evaluation of larvicidal activity of the extracts

The evaluation of the larvicidal activities of the different fractions was done according to method described by Wabo *et al*. (2010). The extract of *D. tripetala* at different concentration (2.5, 1.25, 0.6, 0.3, 0.15 and 0.07 mg/ml) were evaluated for larvicidal effect against *H. bakeri*. Albendazole at the same concentration was used as treated control, while distilled water (5 ml/kg) was used as untreated control. About 200 larvae of *H. bakeri* contained in 200 µl of water was added into each well of a labeled 96-well flat bottom microtitre plate. Thereafter, 200 µl of each concentration of the extracts was added. Each test was done in triplicate. The plates were covered with foil paper and left

at room temperature (25^{o}C) for 24 hours. Thereafter, the content of each well were stirred with pipette, placed on a clean glass slide and examined under the microscope at ×10 magnification. The numbers of larvae that are dead and alive were counted. A larva was considered life if it is motile or move any parts of its body. However if the larva showed no observable motion after 5-10 seconds interval, it was considered dead.

The percentage larvicidal activity was calculated using the formula:

$$\text{Percenage larvicidal activity} = \frac{N-n}{N} \times 100$$

Where "N" = mean number of life larvae found in untreated control mice.

"n" = mean number of life larvae found in treated mice.

3.10 *In vivo* Anthelmintic Screening of the Extracts

The *in vivo* anthelmintic trials of the crude methanol extract (CME), hexane extract (HE), ethylacetate extract (EAE), aqueous methanol extract (AME) and butanol extract (BT) were conducted based on methods described by Suleiman *et al* (2005) and Ngongeh and Fakae (2011). Eighty five mice experimentally infected with *H. bakeri* were randomly allocated into 17 groups of 5 mice each. Mice in groups 1, 2 and 3 were treated with CME at 200, 400 and 800 g/kg respectively. Similarly HE, BT, AME and EAE were tested on mice in groups 4-6, 7-9, 10-12, 13-15, respectively for anthelmintic effect. Mice in groups 16 and 17 were given albendazole (10 mg/kg) and distilled water (5 ml/kg), respectively. All extracts were administered orally on the 16th, 17th and 18th day post-infection while albendazole was administered once.

3.10.1 Faecal egg count reduction test (FECRT)

One gram of faecal sample was collected daily from each group of infected and treated (with extract and albendazole) as well as infected and untreated (distilled water) mice at

day 2, 5 and 7 post treatment (day 20, 23 and 25 post infecton). Modified Mcmaster technique was employed for the faecal egg count. One gram of faeces from each group was homogenized in 12 ml of saturated sodium chloride and sucrose solution of specific gravity of 1.28 and then centrifuged at 5000 *g* for 5 minutes. The supernatant was poured into a labeled sample bottle and the sediment discarded. Aliquot of the supernatant from each of the sample was put into both chambers of McMaster slides and allowed to stand for 5 minutes. The slides were examined microscopically at ×10 magnification. Egg per gram (epg) of faeces was calculated by multiplying the faecal egg count by 40 (Soulsby, 1982; MAFF, 1977).

The anthelmintic effects of each extract of *D. tripetala* fruits was accessed by comparing the EPG of the treated faeces of mice with that of the untreated control mice. The percentage reduction in *H. bakeri* eggs in the treated groups was calculated using the formula described by Abdelqader *et al* (2012):

$$\text{Percentage faecal egg count reduction} = \frac{\text{A-a}}{\text{A}} \times 100$$

Where A= mean faecal egg count in untreated control group (distilled water)

a= mean faecal egg count in the treated group.

3.10.2 Post mortem worm counts

The method described by Ngongeh (2004) was employed for the worm count. On the 25th day post-infection, all the mice used for the *in vivo* anthelmintic trial were deprived of feed but not water for 24 hours so as to empty the gastro-intestinal tract and to make worm counting easier. The mice were euthanized in chloroform chamber the abdominal cavity opened. The entire gastro-intestinal tract was removed immediately and also the

entire length of the small intestine of each mouse was removed and cut longitudinally with a pair of fine surgical scissors. The adult worms were recovered by suspending the small intestine in normal saline. The solution was incubated at room temperature for 20 hours to enable the migration of the adult worm into the solution. The solution was centrifuged and the supernatant discarded. The sediment containing the worms was poured into a petri dish and then observed under a stereomicroscope at ×2 magnification. The adult worms seen were counted and recorded. Furthermore, the intestines were removed from the normal saline and fixed in 10% formalin and then observed under the stereomicroscope at ×2 magnification. The adult worms that did not migrate into the normal saline were counted also. The number of worms that migrated was added to the ones that did not migrate.

The percentage deparasitization was calculated using the formula:

$$\text{Percentage deparasitization} = \frac{W - w}{W} \times 100$$

Where "W" = mean number of worms found in untreated control mice.

"w" = mean number of worms found in treated mice.

Percentage deparasitization of 70 or greater was considered significant.

3.11 Data Analysis

Data obtained from the *in vitro* study were expressed as mean ± standard error of mean (±SEM). Analysis of variance (ANOVA) using GraphPad Prism Version 5 was used to compare the anthelmintic effects of the different fractions of *D. tripetala* fruits extract. The mean percentage activity of the different extract group was compared using Tukey post hoc test. Value of $p < 0.05$ was considered significant.

The *in vivo* activities (fecal egg reduction and deparasitisation rates) were expressed as percentages. Percentage fecal egg count reduction and deparasitization rates of ≥ 70% was considered significant.

CHAPTER FOUR

RESULTS

4.1 Extraction Yield of *D. tripetala* fruits

A brownish, semi-solid crude methanol extract weighing 0.07 % w/w was obtained after extracting 1 kg of the powdered fruits of *D. tripetala*.

The yields of the different portions after serial partitioning of 50g of the crude methanol extract with different solvents are given on the table 4.1.

Table 4.1 Yield, Percentage yield, colour and nature of portions from the crude methanol extract of *D. tripetala* fruits after solvent partitioning.

Extracts	**Yield (grams)**	**Percentage (%) yield**	**Physical nature**	**Colour**
HE	24.87	49.7	Semi-solid	Dark brown
EAE	2.11	4.2	Solid	Brown
BT	3.69	7.4	Solid	Brown
AME	16.80	3.4	Semi-solid	Brown

Key: HE = Hexane extract
EAE = Ethylacetate extract
BT = Butanol extract
AME = Aqueous extract

4.2 Phytochemical Screening of *D. tripetala* Extracts

The metabolites detected in the extracts are presented in table 4.2.

Table 4.2: Qualitative presence of phytochemical constituents in the extracts of *D. tripetala* fruits

Phytoconstituents	**Phytochemical tests**	**Crude methanol Extract**	**Hexane extract**	**Ethylacetate extract**	**Butanol extract**	**Aqeuous methanol extract**
Carbohydrates	Molisch test	+	+	+	+	+
	Fehling test	+	+	+	+	+
Free anthraquinone	Borntrager`s test	-	-	-	-	-
Cardiac glycosides	Kella-killiani test	+	+	+	+	+
Saponins	Frothing test	+	-	+	+	+
Steroids	Lieberman-Burchards test	+	+	+	+	+
Triterpenes	Lieberman-Burchards	+	+	+	+	+
Flavonoids	Shinoda test	+	+	+	+	+
	Sodium hydroxide test	+	+	+	+	+

Table 4.2 continued

Phytoconstituents	Phytochemical tests	Crude methanol Extract	Hexane extract	Ethylacetate extract	Butanol extract	Aqeuous methanol extract
Tannins	Lead subacetate test	+	+	+	+	+
Condensed tannins	Ferric chloride test	+	+	+	+	+
Alkaloids	Mayer`s test	+	+	+	+	+
	Dragendorff`s test	+	+	+	+	+
	Wagner`s test	+	+	+	+	+

Key: + = positive
- = negative

4.3 Acute Toxicity Study of Crude Methanol Extract of *D. Tripetala* Fruits

The Crude Methanol Extract (CME) of *D. tripetala* did not produce death in all the mice given up to 5000 mg/kg. Therefore, the value of $LD_{50} \leq 5000$ mg/kg. The CME at all the doses (10, 100, 1000, 1600, 2900 and 5000 mg/kg) used during the acute toxicity studies produced clinical signs of anorexia and squitting of the eyes in the treated mice. There were no gross lesions observed at postmortem in mice treated with the CME at all the doses (10, 100, 1000, 1600, 2900 and 5000 mg/kg) used for the acute toxicity study as seen on plates I-VI.

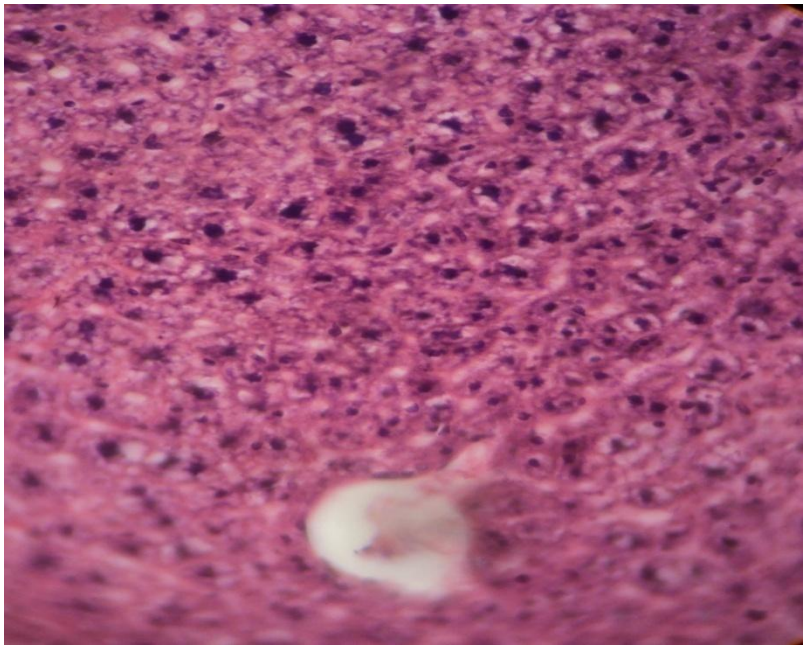


Plate I: Histologic section of liver of mice 3days post treatment with distilled water (5 ml/kg). H and E × 500. No observable pathology

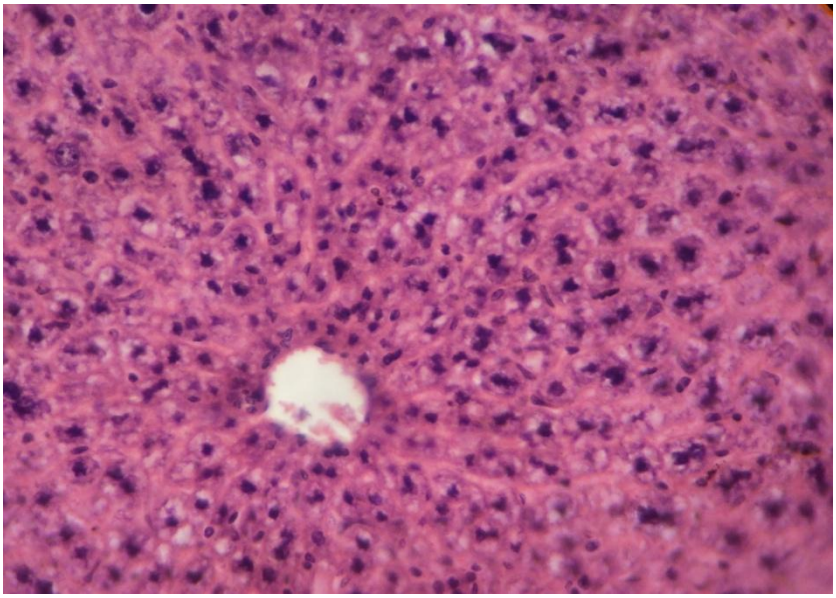


Plate II: Histologic section of liver of mice 3days post treatment with crude methanol extract of *D. tripetala* fruits at 5000 mg/kg. H and E × 500. No observable pathology.

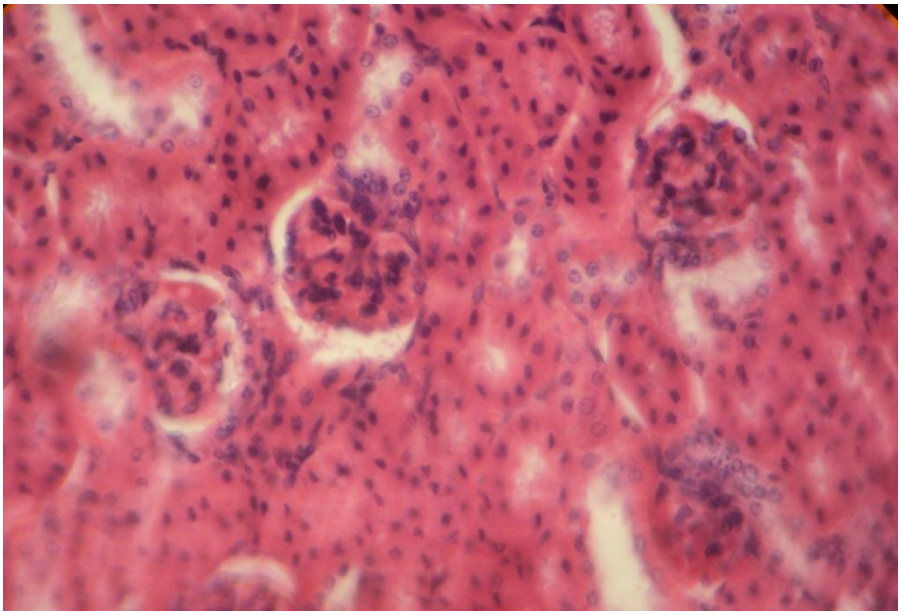


Plate III: Histologic section of kidney of mice 3days post treatment with distilled water (5 ml/kg). H and E × 500. No observable pathology.

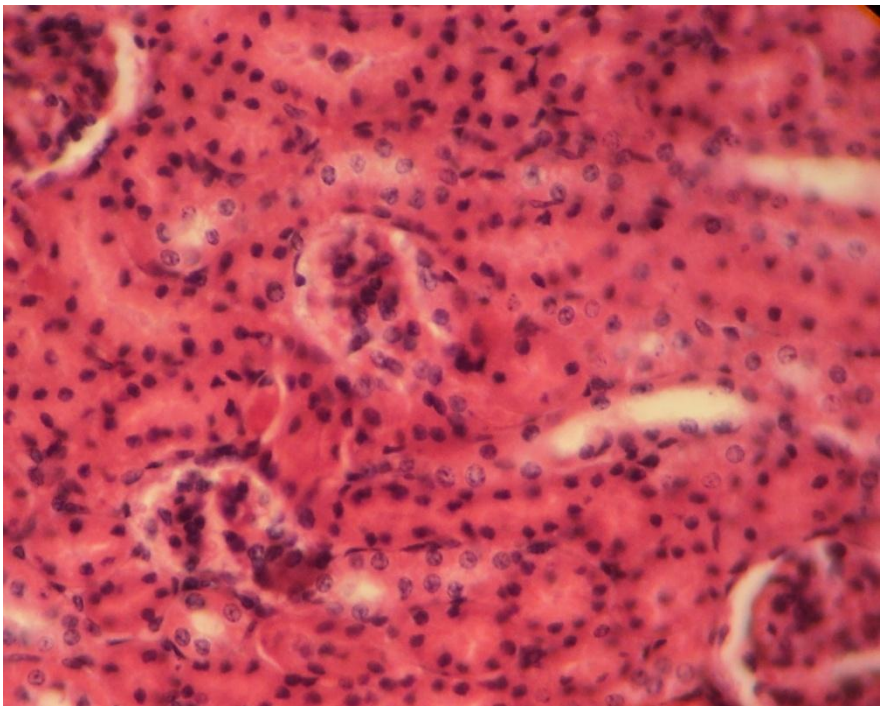


Plate IV: Histologic section of kidney of mice 3days post treatment with crude methanol extract of *D. tripetala* fruits at 5000 mg/kg. H and E × 500. No observable pathology.

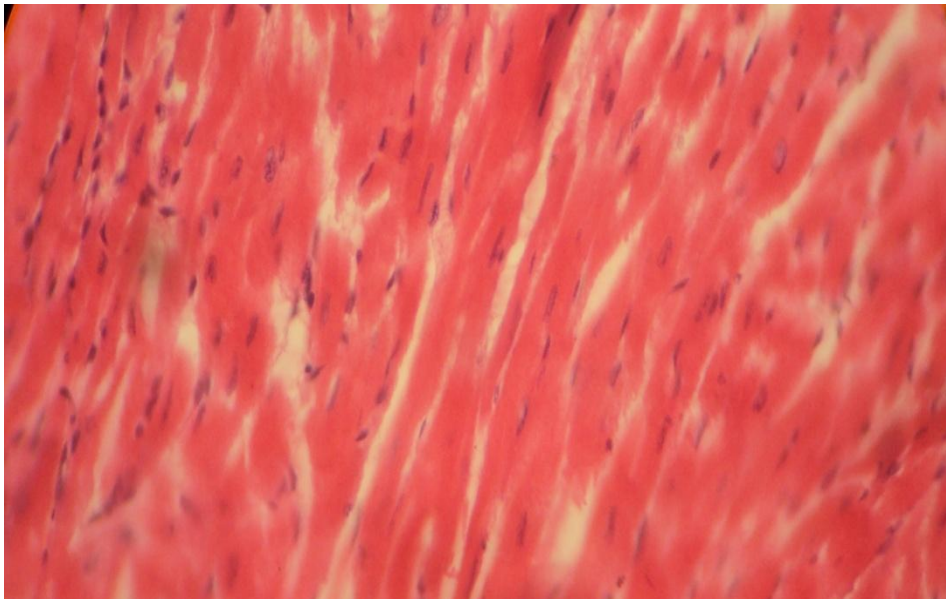


Plate V: Histologic section of the heart of mice treated with distilled water 5 mls/kg. H and E × 500. No observable pathology

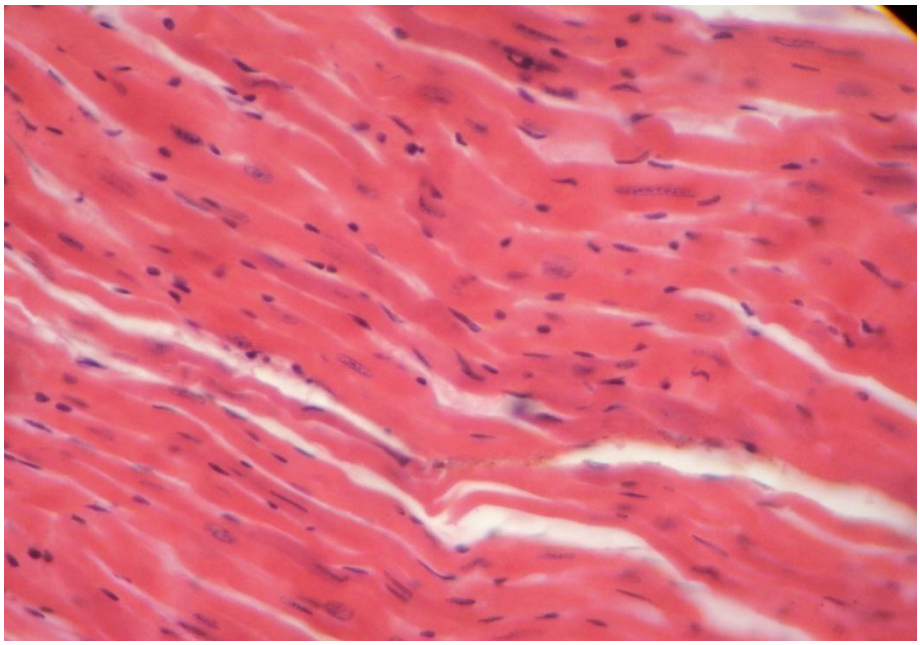


Plate VI: Histologic section of the heart of mice treated with crude methanol extract of *D. tripetala* at 5000 mg/kg. H and E × 500. No observable pathology.

4.4 *In vitro* Anthelmintic Activities of *D.tripetala* Fruits

4.4.1 Egg hatch inhibition

The extracts (CME, EAE, BT and AME) of *D. tripetala* fruits produced significant ($p<0.05$) reduction of egg hatching of *H. bakeri* in a concentration-dependent manner. However, HE was not active against egg hatching of *H. bakeri* when compared to the nontreated (distilled water) control. The result of the egg hatch inhibition of the extracts is shown on table 4.3.

4.4.2 Larvicidal activity

The extracts (CME, EAE, BT and AME) of *D. tripetala* fruits produced significant ($p<0.05$) larvicidal activity against L_1 of *H. bakeri* in a concentration dependent fashion. However, the effect of HE against the L_1 of *H. bakeri* was not significant when compared with the non-treated (distilled water) control. The result of the larvicidal effect of the extracts is shown on table 4.4.

Table 4.3: Effect of *D. tripetala* fruits extracts on egg hatch inhibition.

Extracts	**Concentrations of test substances (mg/ml)**					
Untreated 00.0 mg/ml	**0.07 mg/ml**	**0.15 mg/ml**	**0.3 mg/ml**	**0.6 mg/ml**	**1.25 mg/ml**	**2.5 mg/ml**
			% Mean ± SEM			
CME	14.04±5.15^{c}	61.70±5.28^{b}	65.06±2.58^{b}	84.08±3.79^{a}	88.30±3.02^{a}	93.51±3.19^{a}
AME	16.64±9.78^{c}	47.30±1.69^{c}	52.15±4.31^{b}	74.41±3.21^{b}	82.19±2.90^{a}	87.92±0.51^{a}
BT	6.53±1.07	10.29±1.28^{c}	9.39±0.90	14.27±2.10^{c}	45.61±1.68^{c}	52.40±0.64^{b}
EAE	34.76±0.99^{c}	45.08±2.57^{c}	47.85±5.13^{c}	50.26±6.57^{b}	86.86±1.33^{a}	92.41±1.14^{a}
HE	7.23±0.85	3.99±1.20	6.98±1.51	8.50±1.52	6.07±0.72	5.29±1.15
ABZ	39.83±0.41^{c}	76.03±1.50^{a}	88.25±0.70^{a}	92.13±1.49^{a}	95.59±1.12^{a}	97.17±0.42^{a}
DW 8.45±1.45						

Means with different superscript letters on the same row differ significantly ($p < 0.05$).

Key: CME = Crude methanol extract
AME = Aqeuous methanol extract
BT = Butanol extract
EAE = Ethylacetate extract
ABZ = Albendazole
DW = Distilled water (non treated)

Table 4.4 Larvicidal effect of *D. tripetala* fruit extracts.

Extracts	**Concentrations of test substances (mg/ml)**						
	Untreated 0.00 mg/ml	**0.07mg/ml**	**0.15mg/ml**	**0.3mg/ml**	**0.6mg/ml**	**1.25mg/ml**	**2.5mg/ml**
				% Mean ± SEM			
CME		32.69±3.66^{c}	63.94±1.83^{b}	89.48±2.00^{a}	91.06±2.70^{a}	100±00^{a}	100±00^{a}
AME		38.50±2.98^{c}	50.56±3.78^{b}	54.54±1.26^{b}	82.43±0.91^{a}	82.91±1.77^{a}	85.07±1.35^{a}
BT		29.16±3.11^{c}	58.59±3.59^{b}	61.60±3.53^{b}	63.24±1.19^{b}	71.48±1.20^{b}	77.75±1.96^{a}
EAE		25.88±3.65^{c}	70.03±1.41^{b}	77.56±1.63^{a}	83.17±2.32^{a}	88.17±0.36^{a}	92.62±1.79^{a}
HE		6.03±0.71	6.00±1.36	7.51±2.21	8.19±1.85	6.96±1.77	8.09±2.87
ABZ		78.96±1.58^{a}	84.16±1.95^{a}	96.33±1.28^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
DW	7.13±1.14						

Means with different superscript letters on the same row differ significantly ($p < 0.05$).

Key: CME = Crude methanol extract
AME = Aqeuous methanol extract
BT = Butanol extract
EAE = Ethylacetate extract
ABZ = Albendazole
DW = Distilled water (non treated)

4.4.3 Efficacy of the extracts on egg hatch inhibition

The concentration of each extract of *D. tripetala* that inhibited 50 % (IC_{50}) of *H. bakeri* eggs from hatching as an indication of efficacy was determined from a log concentration-response curve plotted on Microsoft Excel Software (2007) (Appendix III)

The equation of the regression line was used to calculate the concentration of the extract that inhibited 50% (IC_{50}) of *H. bakeri* eggs from hatching. The equation of regression obtained from plot of different concentrations of each extract is presented on table 4.5.

4.4.4 Efficacy of the extracts on larvicidal activity

The concentrations of each extract of *D. tripetala* that killed 50% (LC_{50}) of L_1 of *H. bakeri* as an indication of efficacy was determined from a log concentration response curve plotted on Microsoft Excel Software (2007) (Appendix IV)

The equation of the regression line was used to calculate the concentration of the extract that killed 50% (IC_{50}) of L_1 of *H. bakeri*. The equation of regression obtained from plot of different concentrations of each extract is presented on table 4.6.

Table 4.5: The log concentration-inhibition effect of the extracts of *D. tripetala* fruit against *H. bakeri* eggs

Treatment	**Regression equation**	**Correlation coefficient (R^2)**	**IC_{50} (mg/ml)**
Crude methanol extract	y= 46.09x + 84.78	0.83 (83%)	0.18
Aqeuous methanol extract	y= 44.76x + 76.65	0.94 (94%)	0.25
Butanol extract	y= 31.51x + 35.77	0.74 (74%)	0.28
Ethylacetate extract	y= 38.63x + 75.10	0.80 (80%)	0.22
Albendazole	y= 32.48x + 93.46	0.74 (74%)	0.05

Key: y = 50 %
x (IC_{50}) = concentration of extracts (mg/ml) that inhibited 50 % of eggs of *H. bakeri* from hathing

Note: The IC_{50} is calculated by substituting y (50% egg hatch inhibition) in the regression equation with 50 and making x (concentration of the extract) the subject of the equation.

Table 4.6: The log concentration-larvicidal effect of the extracts of *D. tripetala* fruit against *H. bakeri*.

Treatment	**Regression equation**	**Correlation coefficient (R^2)**	**LC_{50} (mg/ml)**
Crude methanol extract	y=41.42x + 94.78	0.82 (82%)	0.08
Aqeuous methanol extract	y= 33.07x + 77.84	0.89 (89%)	0.14
Butanol extract	y=27.47x+71.37	0.82 (82%)	0.16
Ethylacetate extract	y=38.25x+88.39	0.76 (76%)	0.1
Albendazole	y=25.09x+99.94	0.71 (71%)	0.01

Key: y = 50 %
x (LC_{50}) = concentration of extracts (mg/ml) that killed 50 % of L_1 of *H. bakeri*.

Note: The LC_{50} is calculated by substituting y (50% larvicidal activity) in the regression equation with 50 and making x (concentration of the extract) the subject of the equation.

4.5 *In vivo* Anthelmintic Activity of *D. tripetala* Fruit extracts in Mice

4.5.1 Fecal egg count (FEC) reduction

There were significant reductions in the FEC in mice treated with CME (200, 400 and 800 mg/kg), AME (400 and 800 mg/kg), BT (800 mg/kg), EAE at (200 and 400 mg/kg) and HE at (200 and 800 mg/kg). However, AME (200 mg/kg), BT (200 and 400 mg/kg), EAE (800 mg/kg) and HE (400 mg/kg) had no significant effect on FEC. The FEC reductions rates are presented on table 4.7

4.5.2 Post-mortem worm counts

There were significant reduction of worm counts in mice treated with CME, AME, BT and EAE (200, 400 and 800 mg/kg). Similarly, HE produced significant reduction in worm count (400 and 800 mg/kg). However HE (200 mg/kg) had no significant effect. The worm count reduction rates are presented on table 4.8

Table 4.7 Egg per gram of faeces (EPG) and Percentage fecal egg count reduction rates (% EPGR) in mice (artificially infected with *H. bakeri* treated with *D. tripetala* fruit extracts at different doses.

Extracts	**Doses (mg/Kg)**	**EPG Day 2**	**% EPGR**	**EPG Day 5**	**% EPGR**	**EPG Day 7**	**% EPGR**
Crude methanol extract (CME)	200	11360	0	7360	0	1120	73
	400	8480	0	1600	62	0	100
	800	6720	0	800	81	0	100
Aqueous methanol extract (AME)	200	1760	50	960	77	2560	38
	400	10240	0	1760	58	0	100
	800	15040	0	4640	0	80	98
Butanol extract (BT)	200	4640	0	3040	27	3040	27
	400	2400	32	1600	62	2000	52
	800	4000	0	1760	58	840	80

Table 4.7 continued

Extracts	**Doses (mg/Kg)**	**EPG Day 2**	**% EPGR**	**EPG Day 5**	**% EPGR**	**EPG Day 7**	**% EPGR**
Ethylacetate extract (EAE)	200	3040	14	1440	65	1120	73
	400	3680	0	1600	62	960	77
	800	7520	0	5760	0	1440	65
Hexane extract (HE)	200	800	77	2880	31	1120	73
	400	3040	14	1600	62	1600	62
	800	1440	60	176	96	40	99
Albendazole (ABZ)	10	1280	64	320	92	0	100
Distilled water (DW)	5ml/Kg	3520	0	4160	0	4160	0

Table 4.8 Mean worm counts (±SE) in mice treated with varying doses of *D. tripetala* fruit extract 16 days post infection with 200L_3 of *H. bakeri*.

Extracts Treatment	**Doses (mg/Kg)**	**Mean worm counts (±S.E.M, n=5)**	**Percentage (%) Deparasitization**
Crude methanol extract (CME)	200	7.25±1.25	84
	400	8.75±1.11	80
	800	3.25±1.60	93
Aqueous methanol extract (AME)	200	11.00±1.29	75
	400	6.00±0.91	87
	800	5.25±1.38	88
Butanol extract (BT)	200	7.00±0.91	84
	400	7.75±2.25	83
	800	5.50±0.65	88
Ethylacetate extract (EAE)	200	10.00±2.35	78
	400	6.50±1.04	85
	800	3.00±1.47	93
Hexane extract (HE)	200	16.00±1.58	64
	400	7.25±3.04	84
	800	8.25±3.54	82
Albendazole (ABZ)	10	0.50±0.28	99
Distilled water (DW)	5 ml/kg	44.50±5.24	0

CHAPTER FIVE

DISCUSSION

In this study, methanol was used for the initial extraction because of its ability to extract almost all the chemical components of plants due to its high polarity index (Eloff, 1998). Since almost all of the identified components like tannin, phenol and alkaloids that are active against helminthes are aromatic or saturated organic compounds, they are often obtained through initial extraction with methanol or ethanol (Vilegs *et al*., 1997). The crude methanol extract yield of 7.2% obtained from this study is however; lower than the (14.09%) yield of crude ethanol extract obtained by Ikpi and Nku (2008).

Elekwa *et al* (2011) and Oyemitan *et al* (2013) reported the presence of low polar essential oils in the seeds of *D. tripetala*. The high yield of hexane extract (HE) compared to aqueous methanol extract (AME), ethylacetate extract (EAE), and butanol extract (BT) after partitioning may be attributed to the ability of the hexane to extract the low polar compounds present in the fruits. The variations in the extract yields from using different solvents might be explained by the efficiency of different solvents to extract specific components present in the plant (Aspe and Fernandez, 2011; Tsai *et al*., 2012) and polarity of extracted compounds (Shabbir *et al*., 2011). It could also be due to the availability of the different chemical constituents of the plant (Hsu and Couper, 2006). Factors such as geographical location (Cock, 2011), time of harvest and the method of extraction are also possible sources of variation in the phytochemical yield of plants (Mackie and Mccartney, 1989).

The crude methanol extract of *D. tripetala* fuits were non toxic to mice at a dose of up to 5000 mg/kg when given orally. Most traditional plants when administered to mice orally have been shown to be relatively non-toxic with high median lethal dose (LD_{50}). Suleiman *et al* (2008)

reported that the stem bark extract of *Annona senegalensis* administered orally to mice at a dose of up to 5000 mg/kg were non toxic. Ogbonnia *et al* (2008) also reported the non toxic effect of hydroalcoholic extract of *Xylopia aethiopica* in acute and subchronic toxicity studies. However, Anaga *et al* (2006) reported an LD_{50} of 1120 mg/kg of ethanolic extract of the fruits of *D. tripetala* given intraperitoneally, therefore the high LD_{50} value of *D. tripetala* observed in this study can be attributed to factors that affect oral drug administration, such as denaturation of the extract by gastro-intestinal and liver enzymes; and effect of gastrointestinal pH. All these factors could lower the absorption of the extract into the systemic circulation (Fleisher *et al*., 1999).

During the *in vitro* anthelmintic study, the extracts (CME, AME, EAE and BT) of *D. tripetala* inhibited egg hatching and killed the larvae of *H. bakeri* in a concentration-dependent fashion. This concentration-dependent activity of the extracts agrees with results from several works that have reported increased pharmacological activities with increase in concentration of plant extracts. Suleiman *et al* (2014) reported that extracts of *C. occidentalis* and *G. senegalensis* inhibited *in vitro* hatching and development of *Hemonchus contortus* eggs and larvae in a concentration-dependent manner. Enejoh *et al* (2015a) and Enejoh *et al* (2015b) reported a concentration-dependent ovicidal and larvicidal activity of *Citrus aurantifolia* on *H. bakeri* eggs and larvae. Akther *et al* (2015) also reported concentration-dependent activities of several plant extracts screened for anthelmintic activities *in vitro*. The increase in activity with increase concentration of the extract represent an increase in the bioactive compounds (tannins, saponins, alkaloids, flavonoids) present in the extract that may be responsible for the anthelmintic activity of the fruits.

The extract of *D. tripetala* fruits (CME, BT, EAE and AME) contained carbohydrates, steroids, triterpenes, cardiac glycosides, saponins, tannins, flavanoids and alkaloids which is in agreement

with the findings of Okwu and Ekeke (2003), Ogbonna *et al* (2013) and Adebayo *et al* (2010). However, saponins were absent in the HE. The phytochemical constituents of *D. tripetala* have been suggested to be responsible for the different pharmacological activities of the plant (Reheimann *et al*., 1999; Sparg *et al*., 2004). Hence the phytochemicals detected in the fruit extract during this work could be attributed to the anthelmintic activity demonstrated.

The HE had no significant *in vitro* anthelmintic effect; this may be due to the absence of saponin in the HE, differences in the quality and quantity of the compounds present and varied target site on the parasites (Suresh *et al*., 1997; Amaral *et al*., 1998; Mehmood, 2011).

The slightly higher larvicidal activity of the extracts of *D. tripetala* in this study when compared with the ovicidal effect when comparing IC_{50} and LC_{50} values (Appendix V) may be attributed to the fact that the first stage larvae (L_1) were in their first moult in which the cuticle was not yet hard thus made it easy for the active compounds to penetrate the cuticle and distort metabolism (Enriquez, 1993). Another reason may be due to the resistance of the hard chitinous egg shell to external aggression. The egg shell comprises 3 layers; an external lipoprotein layer, a middle chitinous protein layer and an inner lipid layer. These layers protect the egg from external influences like salt, strong acids and bases, oxidants and reductive agents; and proteolytic compounds. It also serves to prevent evaporation from within the egg and hence dessication (Jimenez-Cisneros, 2007).

In the *in vivo* study, the faecal egg count (FEC) reduction observed following treatment with the extract indicated the anthelmintic potential of *D. tripetala*. This was clearly demonstrated by the ability of the extracts to cause deparasitization as seen during the post mortem worm count (PMWC). Mice in the un-treated control (distilled water) had significantly ($p < 0.05$) higher FEC

and PMWC compared with rats treated with the extracts. The percentage deparasitization of 50% or more observed in this study, showed that the extracts administered at different doses (200, 400 and 800 mg/kg) were effective against *H. bakeri* in mice. As an anthelmintic is considered effective only when it can reduce 50% or more of the worm burden in an infected animal (Suleiman *et al*., 2005). AME and EAE produced dose-dependent deparasitization. Studies have shown that different plant extracts when tested for *in vivo* anthelmintic activity, produce their effect in a dose-dependent manner (Ademola *et al*., 2004; Ngonge and Fakae, 2011; Giri *et al*., 2015). Although in this study, CME, BT and HE did not show dose-dependent activities. This may be attributed to physiology of the individual animals such as activities of enzymes, blood volume and hormonal balance (Testa and Krämer, 2006) that may affect transportation, bioavailability, degradation and storage during metabolism of the drug (extract) *in vivo* (Wesche and Barnish, 1994).

Though the active principle responsible for the anthelmintic activity of *D. tripetala* fruit is not yet known, bioactive compounds such as tannins, saponins and alkaloids present in the plant as revealed during phytochemical analysis might be responsible for the anthelmintic activity of the plant.

Tannins and their metabolites have been shown to have a direct effect on the viability of the preparasitic stages of helminths (Duncan, 1996; Williams *et al*., 2014; Dhakal *et al*., 2015). The possible anthelmintic effect of tannins attributed to their ability to interfere with energy generation by uncoupling oxidative phosphorylation (Athanasiadou *et al*., 2000). Tannins can also bind to free proteins in the gastrointestinal tract of the host animal (Athanasiadou *et al*., 2007) or glycoproteins on the cuticle of the parasite (Thompson and Geary, 1995) and cause death. However, this effect of tannins depends on the type and content of tannins in the plant

(Athanasiadou *et al*., 2007). Plants with higher content of condensed tannins have been shown to have a superior anthelmintic activity compared to those with low amount of condensed tannins (Brunett and Hoste, 2006; Laerke and Hedemann, 2012).

Saponin has been shown to be responsible for the anthelmintic activity of some medicinal plants (Mc Allister *et al*., 2001). Monodesmoside saponin act by destabilizing the membrane and increasing the cell permeability of helminthes parasites (Price *et al*., 1987; Gee and Johnson, 1988). Saponins present in many medicinal plants enhance the absorption from the gastro-intestinal tract and activity of other phytochemicals and substances present in the plants (Nguedia *et al*., 2004).

Simarly, alkaloids present in some plants were demonstrated to have anthelmintic effect. Alkaloids extracted and isolated from the stem bark of *Afzelia Africana* demonstrated a significant anthelmintic activity in rats infected with *Nippostrongylus braziliensis* (Nafanda and Obenta, 2012). Lateef *et al* (2003) reported that alkaloid extracted from the root of *Adhatoda vestica* were effective against mixed gastrointestinal helminth infections in sheep. Also, Al-Qawari *et al*., (2001) reported that alkaloids extracted from both the latex and leaves of *Calotropis procera* were effective in inhibiting the exsheatment of L_3 of *Hemonchus contortus* to L_4 in sheep.

From this study, there was a slight variation between the *in vitro* and *in vivo* anthelmintic activity of *D. tripetala* fruit extracts (CME, EAE, AME and BT). For instance in the *in vitro* anthelmintic test, CME showed as high as 100% larvicidal activity at concentrations of 1.25 and 2.5 mg/ml (0.4 mg/ 0.32 ml and 0.8 mg/ 0.32ml respectively) while in the *in vivo* study, the percentage deparsitization at 400 and 800 mg/kg (8 mg/20 g and 16 mg/20 g of mice respectively which is

equivalent to 8 mg/0.32 ml and 16 mg/0.32 ml in concentrations) were 80% and 93% respectively. The differences between *in vitro* and *in vivo* efficacies could be attributed to factors associated with drug absorption in the gastro-intestinal tract environment as well as the physiology (activities of enzymes, hormonal balance and genetics factors) of the animals which may alter the helminth's response to drugs. Also, several factors affect oral drug administration such as destruction of the drug by gastro-intestinal and liver enzymes, low gastric pH and formation of drug complexes with food. These might have reduced the efficacy of the extracts *in vivo.* In the *in vitro* study, the extracts had direct contact with the parasites resulting in a slightly higher efficacy. Other workers have reported similar discrepanies between the *in vitro* and *in vivo* anthelmintic activity of medicinal plants (Alawa *et al*., 2003; Iqbal *et al*., 2004; Adedapo *et al*., 2007; Ademola and Eloff, 2011).

Although, HE did not show *in vitro* anthelmintic effect, however, the extract was active when tested *in vivo*. Perhaps the activity of the extract *in vivo* could be that the bioactive compound(s) present in the extract were converted to an active metabolite *in vivo* by a mechanism that is not known. Studies have shown that some drugs that are inactive when tested *in vitro* showed activity when administered into the animal's body. The conversion of the inactive drug to active metabolite is usually done by some enzymes systems present in the body (Diwaker and Jadon, 2011).

Albendazole showed a higher anthelmintic activity than all the extracts of *D.tripetala* fruit extracts. This higher anthelmintic activity of albendazole may be attributed to its purity when compared to the crude extracts. Albendazole is broad-spectrum anthelmintics; and it has been

shown to possess both *in vivo* and *in vitro* anthelmintic activity and is used as a conventional anthelmintic agent (Riviere and Papich, 2009). Perhaps if the active compound(s) such as tannins, alkaloids, saponins, flavonoids contained in the extracts of *D. tripetala* fruits are isolated, the extracts could possess a much higher anthelmintic effect.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The crude methanol extract of *D. tripetala* fruits is non-toxic and safe with oral route LD_{50} of $\leq$ 5000 mg/kg.

The preliminary phytochemical screening of the extracts showed that the plant contained carbohydrate, glycosides, steroids, flavonoids, alkaloids, saponins, triterpenes and condensed tannins, some of which might be responsible for the anthelmintic activity of the plant.

The findings from this study suggest that the extracts (crude methanol, aqueous methanol, hexane, ethylacetate and butanol extracts) of *D. tripetala* fruits possess anthelmintic activity *in vitro* and *in vivo* except hexane extract which did not show appreciable effect on *in vitro* studies. The *in vitro* and *in vivo* anthelmintic activities of the extract are comparable to that of albendazole. Since *D. tripetala* is considered as quality food with good medicinal properties, it can serve as a potent anthelmintic and nutritious food supplements for humans and animals.

6.2 Recomendations

1. There is the need to isolate and characterize the active principles from the extracts of *D.tripetala* fruit. This could perhaps provide a potent and safe anthelmintic drug.

2. *In vivo* studies in field trials and laboratory studies involving the target helminth parasites that affect domestic animals should be conducted to evaluate the efficacy of this plant in those animals.

3. Further detailed chronic toxicity studies involving other routes of administration (such as intraperitoneal route) of the extracts in order to establish their safety should be conducted

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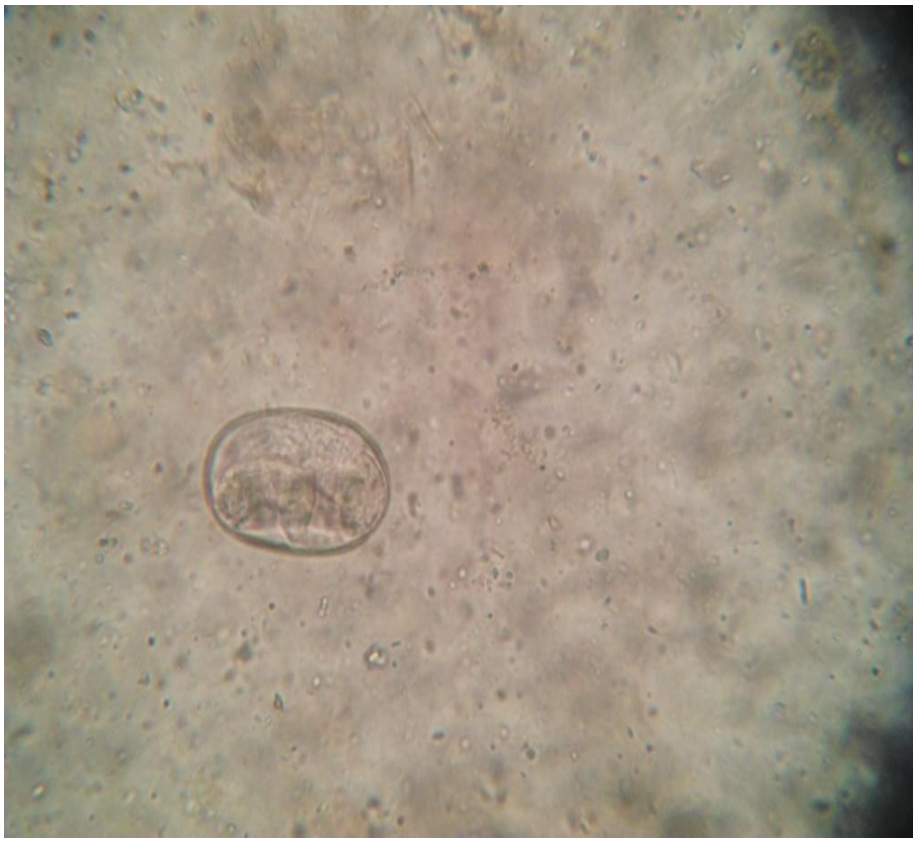
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APPENDICES

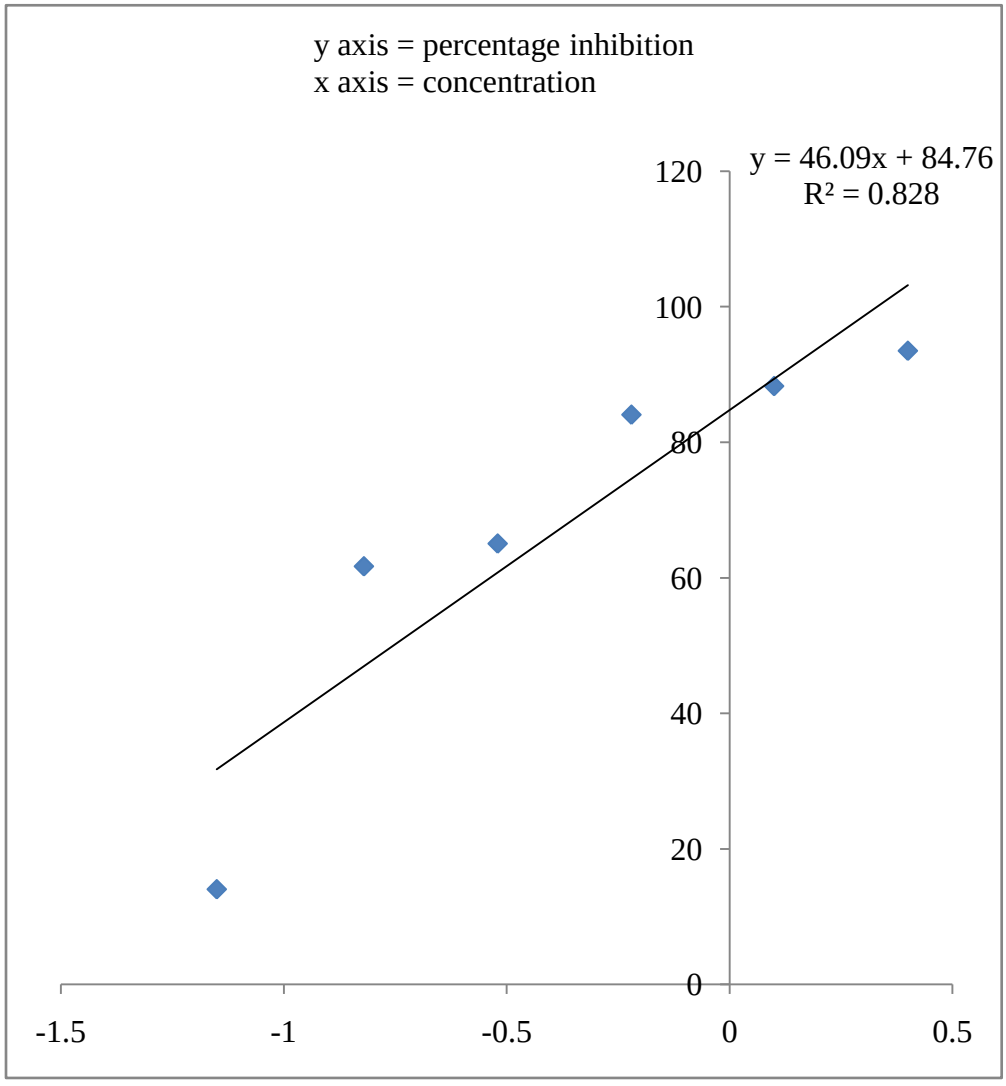
Appendix I: Embryonated egg of *H. bakeri* × 10 magnification.



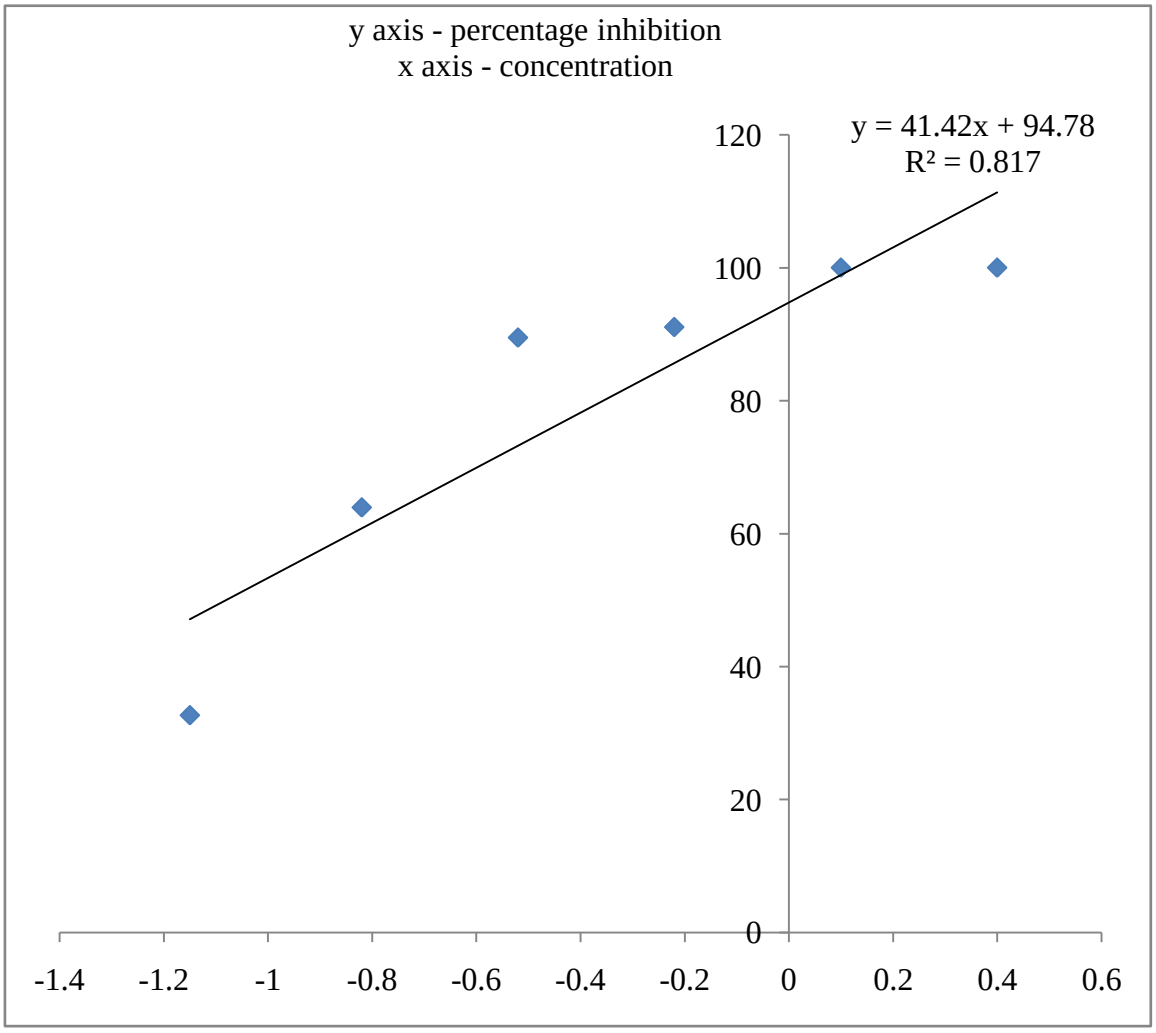
Appendix II: Third stage larvae of H. bakeri ×10 magnification



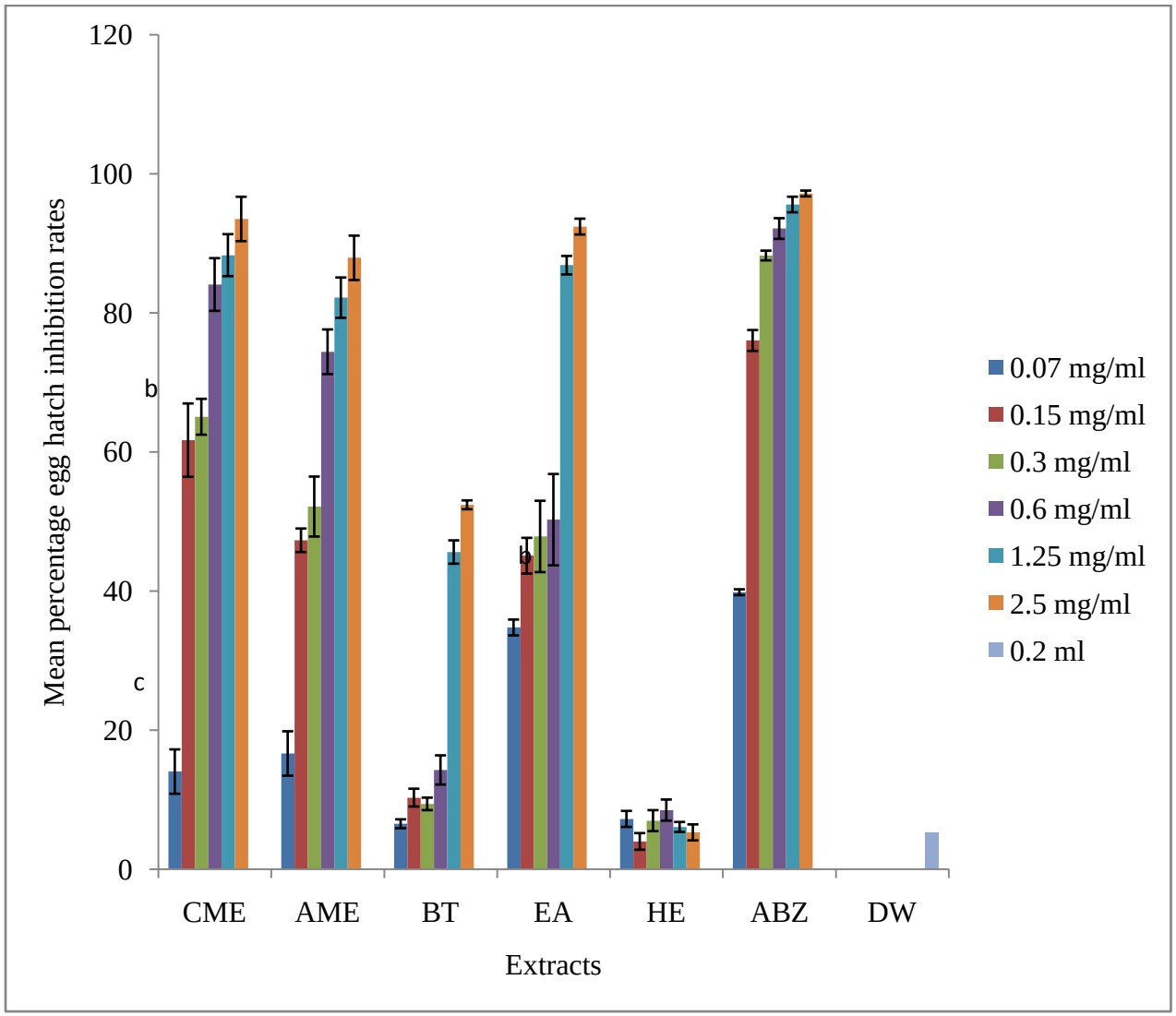
Appendix III: The log concentration response curve for CME showing the pattern of inhibition of *H. bakeri* eggs.



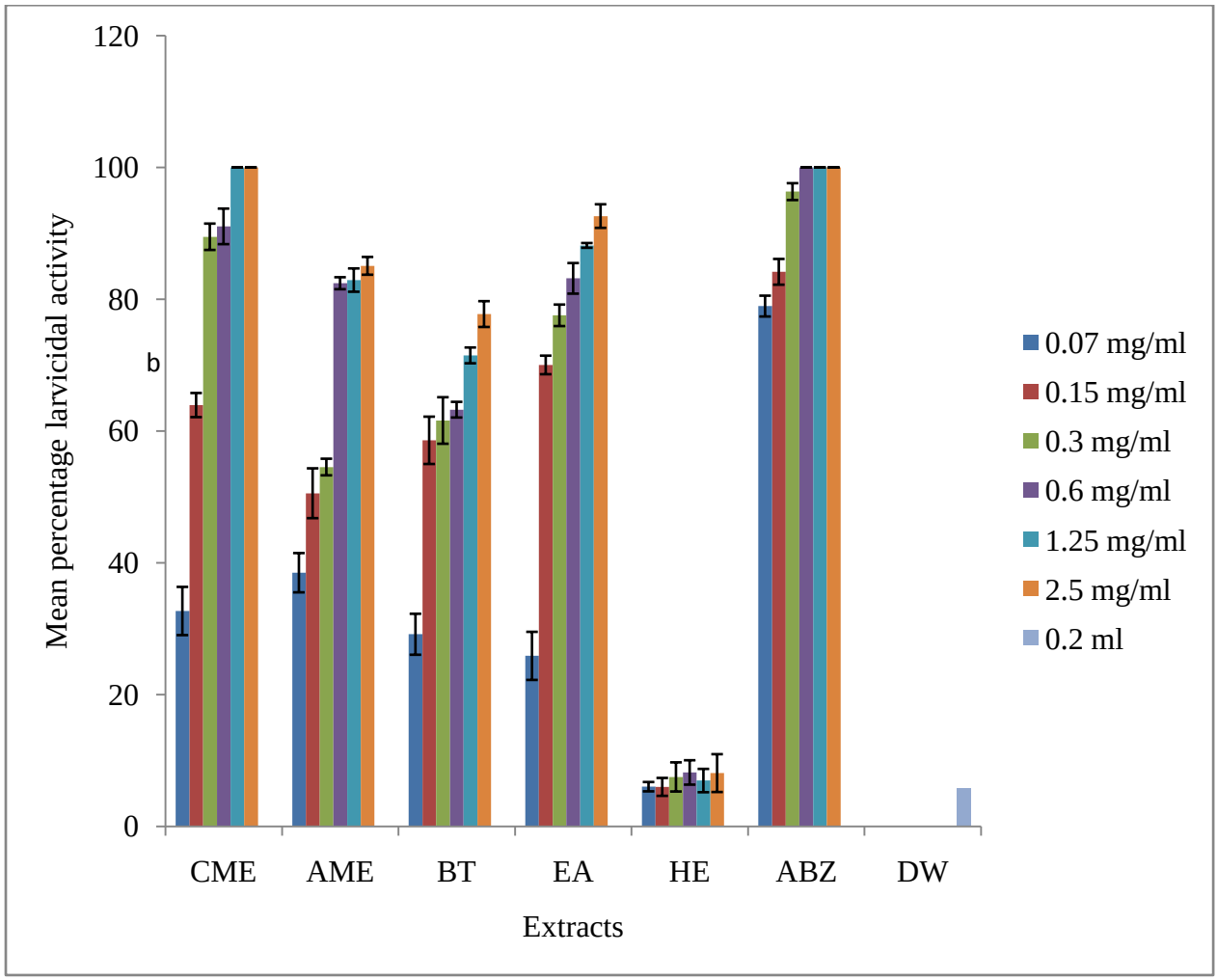
Appendix IV: The log concentration response curve for CME showing the pattern of larvicidal activity.



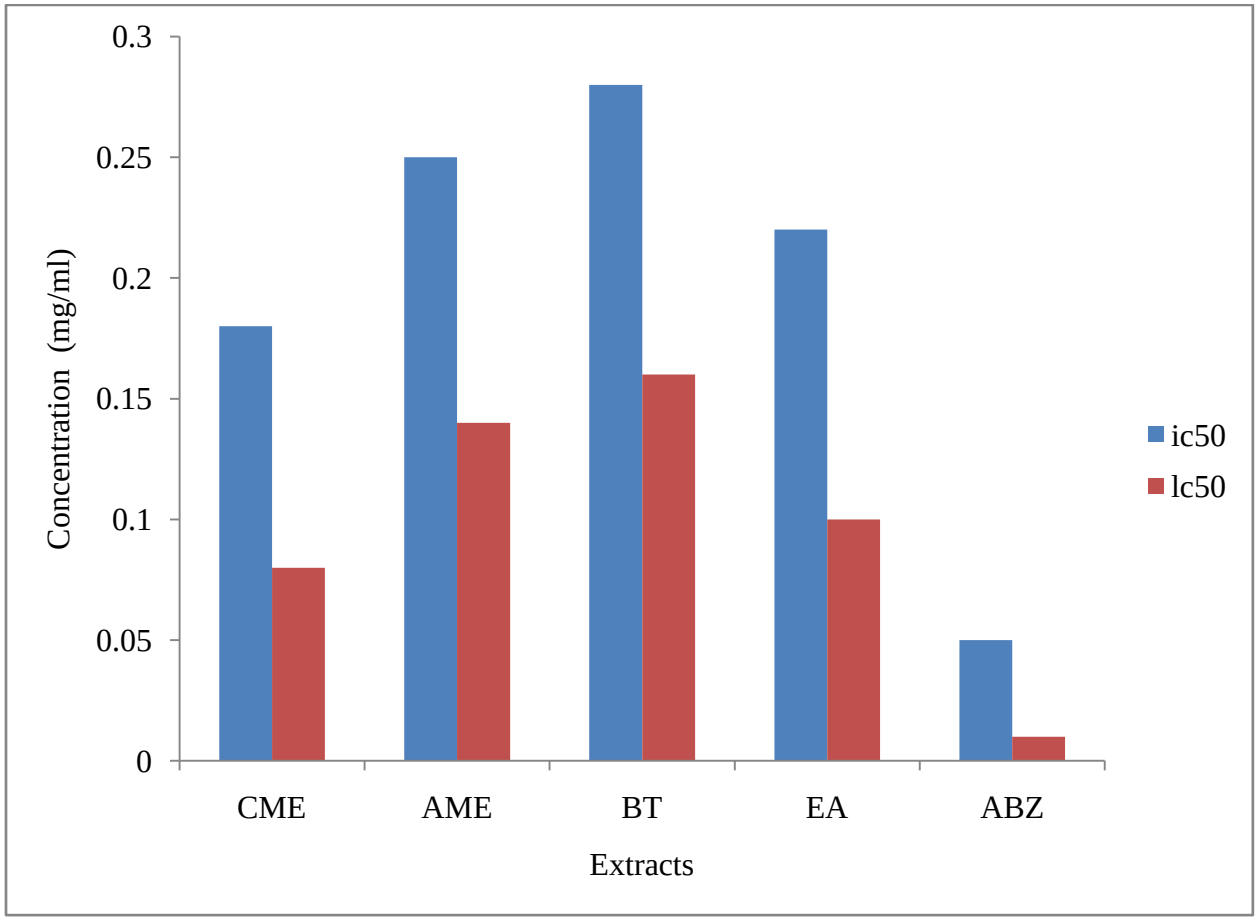
Appendix V: Mean ± S.E.M percentage egg hatch inhibition of *H. bakeri* by crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BT), ethylacetate extract (EA), hexane extract (HE) of *D. tripetala* fruits and albendazole (ABZ) at varying concentrations.



Appendix VI:Mean ± S.E.M percentage larvicidal effect of crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BT), ethylacetate extract (EA) hexane extract (HE) *D. tripetala* fruit and albendazole (ABZ) at varying concentrations.



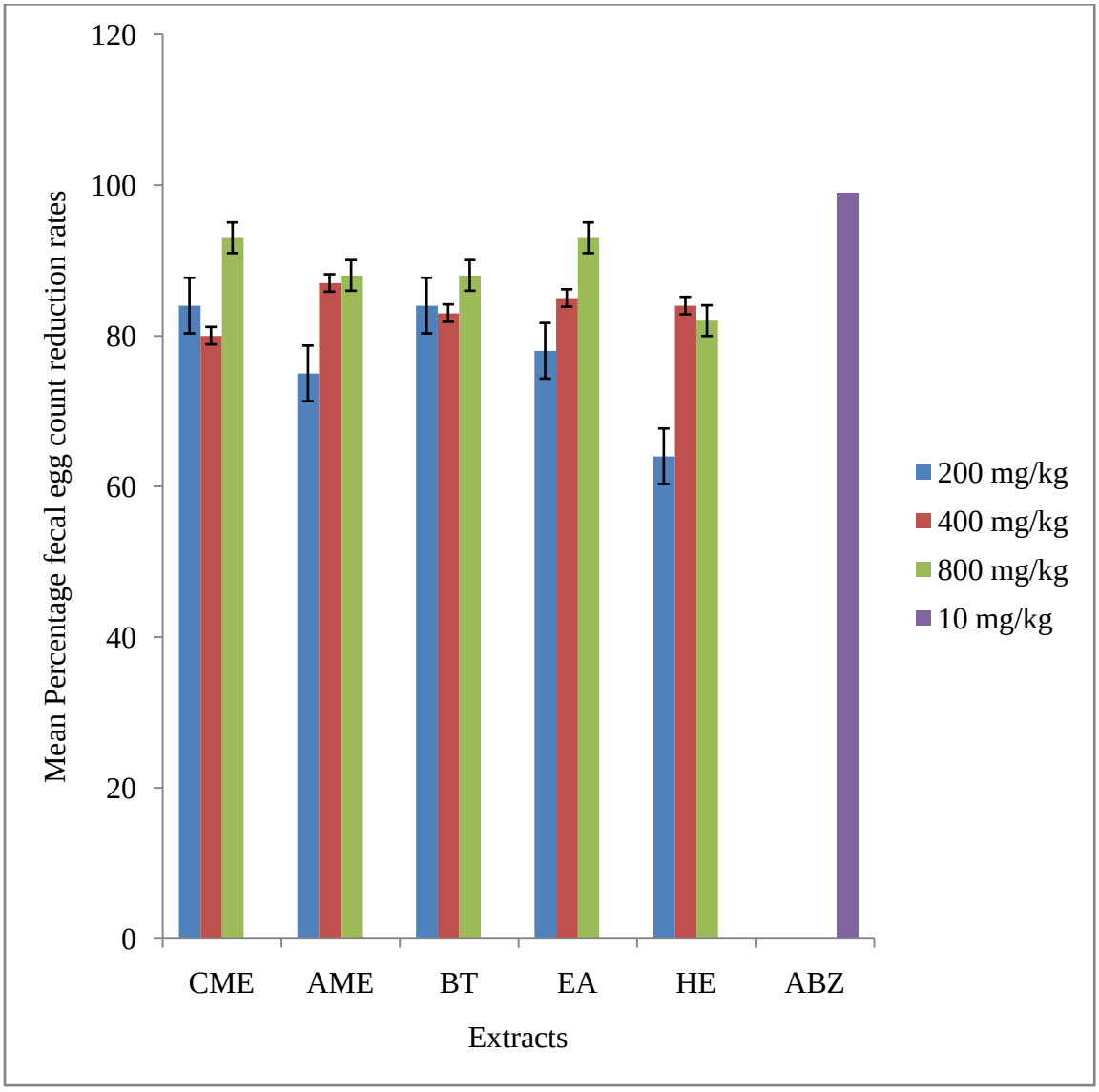
Appendix VII: IC50 and LC50 values of *D. tripetala* fruits against *H. bakeri*.



Key: IC50: The concentrations of the extracts that inhibited 50 % of *H. bakeri* eggs from hatching
LC50: The concentrations of the extracts that killed 50 % of the larvae
CME: crude methanol extract.
AME: aqueous methanol extract.
BT: butanol extract.
EA: ethylacetate extract.
ABZ: albendazole.

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Appendix VIII: Percentage fecal egg count reduction rates (% EPGR) in mice (artificially infected with *H. bakeri*) treated with crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BT), ethylacetate extract (EA), hexane extract (HE) of *D. tripetala* fruit at different doses and albendazole (ABZ) at 7 days post treatment.



Appendix IX: Mean percentage worm counts (±SE) in mice (artificially infected with 200L_3 of *H. bakeri*) treated with crude methanol extract (CME), aqueous methanol extract (AME), butanol extract (BT), ethylacetate extract (EA), hexane extract (HE) of *D. tripetala* fruit at varying doses and albendazole.

