NEMATOCIDAL ACTIVITY OF METHANOLIC EXTRACTS OF LEAF, STEM BARK AND ROOT OF *AZADIRACHTA INDICA A. JUSS. (*NEEM) AGAINST *HAEMONCHUS CONTORTUS*(RUDOLPHI 1803) COBB 1898

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AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA

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DEPARTMENT OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA

JANUAURY, 2014

DECLARATION

I hereby declare that this thesis titled **"**Nematocidal activity of methanolic extracts of leaf, stembark and root of *Azadirachtaindica* (neem) against *Haemonchuscontortus"*was performed by me in the Department of Biological Sciences, under the supervisionof Prof. P. A. Audu and Dr. E. Kogi.The information derived from the literature has been duly acknowledged in the text, and a list of references provided. To the best of my knowledge and belief, no part of this thesis was previously presented for another degree or diploma at any University.

---------- ----------

JATAU, NAOMI **DATE**

CERTIFICATION

This research thesis titled Nematocidal activity of methanolic extracts of leaf, stembark and root of *Azadirachtaindica*(neem) against *Haemonchuscontortus*meets the regulations governing the award of degree of Master of Science (M.Sc.) of Ahmadu Bello University, Zaria, and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty my greatest sustainer who made this work a success, and in memories of my beloved father and sister; Late MrJatauTsaku and Late Sarah Jatau. May your gentle souls rest in perfect peace. Amen.

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ABSTRACT

The antihelminthic activity of methanolic extracts of leaf, stembark and root of *Azadirachtaindica* were evaluated *in vitro* against*Haemonchuscontortus* larvae. Phytochemical screening conducted on the extracts of all the plant parts revealed the presence of carbohydrates, cardiac glycoside, saponins, flavonoids, tannins and alkaloids, leaf had steroids, while stembark and root had triterpenes. The quantitative estimation of the phytochemical constituents revealed a high percentage in flavonoids followed by alkaloids and tannins with least quantitative percentages insaponins: 20.21, 27.50, 20.95; 5.25, 5.48, 5.48; 4.95, 4.93, 4.15 and 0.75, 0.68, 0.67respectively. Eggs of *H.contortus* were cultured in culture plates maintained at 27°C in an incubator. The infective third stage larvae (L3) were recovered from 7-9 day old sterilefaecal cultures. The larvae harvested were concentrated at 1000 rpm for 15 minutes, 0.1 ml of the larvae containing 120 L3 were put into wells of microtitre plate and concentrations of each of the extracts (leaf, stembark and root) at 0.1mg/ml, 1.0mg/ml, 10.0mg/ml, 100mg/ml, negative control (water) and positive control (Levamisole) were added to each of the wells with six replication. After addition of the treatments, it was viewed under the microscope once in 6 hours for a period of 48 hours and the number of mortality recorded. The lethal concentration (LC50/ EC) value for the leafis 12.30mgml^{-1}, 12.58mgml^{-1} for the stem bark and 15.84mgml^{-1} for the root extract. Mortalities of the parasites increased with increase in the concentration and with the time of exposure. At the peak time of exposure

(48 hours) and at the highest concentration of 100mgml^{-1}, mortalities were higher than those of the least time of exposure (6 hours) and with the lowest concentration of 0.1mgml^{-1}. The data showed highly significant differences between the plant parts, time of exposure, concentration of the extracts, the time of exposure and concentration and between the plant parts and concentration ($p=0.001$). Although, mortality of the parasites increased with increase in time, there were no significant differences between the plant parts and the time of exposure ($p=0.92$) and between the plant parts, time of exposure and concentration ($p=0.99$).Mortalities recorded were high in the positive control wells with increase in mortality as the time of exposure increased but in the negative control wells, mortalities were not recorded. The study concludes that mortalities recorded were due to the effects of the extracts on the parasites. Validation of the efficacy of the extracts of this plant is suggested to determine the effects of natural or experimental haemonchosis in ruminants.

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CHAPTER ONE

1.0 Introduction

*Azadirachta indica,*commonly known as neem in English and Dogonyaro in Hausa,is an evergreentree in the Mahogany family Meliaceae. It is native to India, Pakistan and Burma, growing in tropical and semi-tropical regions (Balakrishnan *et al.*, 2007).

It is the most versatile, multifarious tree of the tropics with immense potential growing to about 25m in height with semi-straight to straight trunk, 3m in girth and spreading branches forming a broad crown (Kumar and Gupta,2002).

It possesses useful non-wood products (leaves, bark, flowers, fruits, seed, gum, oil and neem cake) than any other tree species. These non-wood products are known to have antidermatic, antifeedent, antifungal, anti-inflammatory antipyorrhoeic, antiscabic, cardiac, diuretic, insecticidal, larvicidal, nematocidal, spermicidal and other biological activities (Brahmachari,2004). The wider application and activities of neem have made it a green treasure (Khanna, 1992,Suri and Mehrotra 1994).

The tree has adaptability to a wide range of climatic, topographic and edaphic factors. It thrives well in dry, stony shallow soils and even on hard calcareous soils or clay pan at a shallow depth (Koul *et al.*,1990,Schmutterer, 1990).

Neem tree requires little water and plenty of sunlight (Anonymous,2006, Sateesh,1998). The tree grows naturally in areas where the rainfall is in the range of 450 to 1200 mm.

However, it has been introduced successfully even in areas where the rainfall is as low as 150 to 250 mm. It grows on altitudes up to 1500 m (Chari, 1996, Jattan *et al.,* 1995, Tewari, 1992).

It can grow well in wide temperature range of 0^0C and 49^0C (Hegde,1995). It cannot withstand water-logged areas and poorly drained soils. The pH range for the growth of neem tree lies in between 4 to 10. It grows on almost all types of soil including clayey, saline and alkaline soil but it does well on black cotton soils and deep well drained soils with good sub-soil water. Neem tree has the ability to neutralize acidic soils by a unique property of calcium mining (Hegde, 1995).

The roundworm,*Haemonchus contortus* (Rudolphi 1803, Cobb 1898) which is also known as red stomach worm, wire worm or Barber's pole worm, is a very common parasite and one of the most pathogenic nematode of ruminants (Burke, 2005). In domestic animals, this parasite is a source of great economic loss (Hickman, 2008).

It is a highly pathogenic parasitic nematode that affects large number of wild and domesticated ruminant species and is the most economically important parasite of sheep and goats worldwide. It is responsible for 80% of worm infections in small ruminants (Balakrishnan *et al.,* 2007). This worm is a worldwide threat, but it is more prevalent in sub-temperate and temperate regions under warm and wet conditions (Fleming, 2006).

Haemonchus contortus is cylindricall shaped, tapered at both ends and has a complete digestive system. Adult worms are blood feeders that reside in the abomasum (stomach)

and are approximately 2cm in length when mature(Roberts and Janovy, 2000). They are dioecious with single females typically producing several thousand eggs (5000-10,000) per day which pass out of the host in faeces and develop to infective L3 larvae on the pasture (Merck, 1998; Roberts and Janovy, 2000; White and Newton, 2001).

H. contortus is a member of the superfamily Trichostrongyloidea (Strongylida) which contains most of the economically important parasitic nematodes of grazing livestock. *H. contortus* cost the global livestock industry billions of dollars per annum in lost production and drug costs. The major problem lies within the agricultural industry. The parasites cause great economic losses in domestic animals, specifically sheep, cattle and goat.*Haemonchus contortus*being a blood sucker, it can induce anaemia and oedema. Also, the haemolytic proteins that the parasite releases can lead to other intestinal disturbances. The host will often die of major disorders such as diarrhea, severe dehydration and severe blood loss.

Haemonchus contortus is known to adapt well to harsh conditions, which makes it more difficult to eliminate (Jacquiet *et al*., 1998; White and Newton, 2001). Resistance to all the major antihelminthic classes is common worldwide often leading to failure of treatment and control.

1.1 Statement of ResearchProblem

Control of nematode infections has traditionally been done using antihelminthics drugs (chemotherapy) with best results obtained when this approach is initiated with proper grazing management and resistant animals. However, in the last two to three decades there has been over dependency and even misuse of the chemotherapeutic approach with consequent evolution of antihelminthic resistance (Ngomuo *et al*., 1990; Prichard, 1994). Apart from antihelminthic resistance, poor availability and affordability of antihelminthics to resource-poor farmers in developing countries have compounded the problem (Hammond *et al.,* 1997). Moreover, there is growing concern over drug residues in the food chain and environment. Search for novel antihelminthics that are more suitable and less toxic is undoubtedly a sensible approach to the control of parasitic infections. One such alternative could be harnessing the available ethno botanical knowledge (Hammond *et al*., 1997), i.e., the use of medicinal plants with antihelminthic activity. Plantbased antihelminthics have been known and used in many parts of the world for a long time but little research has been done to validate their use, especially in veterinary medicine.

1.2 Justification

Three approaches to the control of nematodiasis are; chemotherapy, chemoprophylaxis and vector control. Attempts to develop a vaccine against nematodes have been dwarfed by the parasite's ability to avoid total detection by the host's immune system and in some cases, there are drug resistance problems.

Deficiencies such as lack of efficacy of antihelminthic drugs against all stages of the diseases, the need for parenteral administration and the inability to eliminate all strains of species of nematodes make treatment costly and difficult. Furthermore, the methods used in the control of insect/vectors are either expensive or very tedious.

In view of the problems presented by present nematocides, there is therefore an urgent need to source for suitable alternative drugs in order to combat the disease. Plants used in indigenous medicine are considered to be potential sources for the development of alternative therapeutics (Cox and Blick, 1994). Since herbal treatment for various diseases in Africa is still widespread practice (Anokbongo,1992), an ethno-botanical approach through collaboration with traditional healers may prove to be a rich source of drug discovery. These local knowledge systems are reported to be as effective and cheaper than their orthodox equivalents (Sibanda and Okoh 2008). On the other hand, herbal remedies are the only source of day to day health care for a large number of the world's population in rural areas. Therefore, it is vital to identify those plants which are

suitable for use, focusing on the efficacy and less toxicity. Natural products from plant materials are increasingly being explored for the extraction of bioactive agents for the development of drugs against diseases. Different regions of Ngeria are affected by nematode vectors hence endemic for nematodiasis. In this light, there is the need to create and enrich the pool of information on plants with medicinal value which will serve as a referral in the future for the development of drugs against several pathogen causing diseases.

1.3 Aim

To evaluate the potency of methanolic extracts of*Azadirachta indica*(neem) against *Haemonchus contortus.*

1.4 Hypotheses

i. There is no significant difference between the effects of different concentrations of methanolic extracts of the leaf, stembark and root of *Azadirachta indica* against *Haemonchus contortus/*ere is no significant difference between the time of exposure of *Haemonchus contortus* to the variousplant parts extracts

1.5 Objectives

i. To determine and compare the effects of different concentrations of methanolic extracts of leaf, stem bark and root of *Azadirachta indica*(neem) against *Haemonchus contortus*.

ii. To determine the effect of time of exposure of the various plant parts extracts against *Haemonchus contortus*.

CHAPTER TWO

2.0 Literature Review

2.1 The plant: *Azadirachta indica*

2.1.1 Background of the Plant

Medicinal plants have been used as folklore remedies over the years to treat, manage or control man's ailments. They contain large varieties of chemical substances that possess important therapeutic properties used in the treatment of these ailments. Also, the problem of bacterial resistance to commonly used antibiotics has necessitated the search for newer and alternative compounds for the treatment of drug resistant infections and the high cost of conventional drugs, particularly in resource poor communities of the African continent has led to the increased use of plants as an alternative for the treatment of infectious diseases (Sibanda and Okoh 2008). Several findings on the chemotherapeutic potentials of plants have shown that they can be sources of antimicrobial compounds of value (Rabe and van Staden, 1997).

The neem tree has been described as *A. indica* as early as 1830 by De Jussieu and its taxonomic position is as follows:

Order Rutales

Suborder Rutinae

Family Meliaceae (mahogany family)

Subfamily Melioideae

Tribe Melieae

Genus *Azadirachta*

Species *indica*

Itis indigenous to arid regionsof southern and southeasternAsia**,** but is now widespread inadjacent countries including Bangladesh, Pakistan**,** Sir Lanka**,** Indonesia, Malaysia, Thailand, and Burma (Sombatsiri *et al.,* 1995). Duringthe last 150 yearsthe tree has been introducedinto Africaand is nowconcentrated in a belt stretching across the Africancontinent from Somalia to Mauritania;and in semi arid areasbetween 10:14^{o}N E. It is also propagated on islands in the South Pacific, in Australia, in several countries in the Caribbean, andin Central and South America (Ascher, 1993).

The tree is a fast growing plant which may reach a height of 25 m:it is bestgrown under tropical conditions and cantolerate an extended dry season, severe droughts and poor shallow soils (Koul *et al.,* 1990; Schmutterer, 1990), butis susceptible to excessive frost (Koul *et al.,*1990).

Itis propagated easily by seed and 9 to 12 months old seedlings transplant well. The fruits, the main source of pesticide products are produced on drooping panicles, usually once a year.Seed is normally produced after about five yearsand after this time 37 to 50kilograms of seed per tree are produced every year (Koul *et al.,*1990).

2.1.2 Origin and Distribution of Neem

Two species of *Azadirachta* have been reported, *Azadirachta indica A. juss* – native to India sub-continent and *Azadirachta excelsa kack*. – confined to Philippines and Indonesia (`Jattan *et al.,* 1995, Tewari, 1992). The former grows as a wild tree in India, Bangladesh, Burma, Pakistan, Sri Lanka, Malaysia, Thailand and Indonesia. Presently neem tree can be seen growing successfully in about 72 countries world-wide, in Asia, Africa, Australia, North, Central and South America (Sateesh, 1998; Hegde, 1995; Suri and Mehrotra, 1994).

2.1.3 Chemistry of Neem

Neem posseses array of biologically active compounds which arechemically diverse and structurally complex.

Neem chemistry dates back to 1880-90 when influenced by its folk-lore medicinalvalues. The Indian scientist Siddiqui was the first scientist to bring the plant to the attention of phytopharmacologists in 1942 while working at the Scientific and Industrial Research Laboratory at Delhi University, British India. He took up the isolation of active ingredients from its seed and other parts and isolated three bitter compounds from neem oil, which he named ninbin, ninbinin and ninbidin respectively (Ganguli, 2002). The seeds contain a complex secondary metabolite azadirachtin.

The neem constituents belonging to chemically diverse classes have been dividedinto two major groups: Isoprenoids and Non-Isoprinoids. The later categorycomprises glycerides, polysaccharides, sulphurones compounds, flavonoids and theirglycosides.

I. ISOPRENOIDS:

i) Diterpenoids: 24 compounds of this class have been isolated from root and stem barkof neem. These chiefly belongs to two groups – **podocarpanoids** and **abietanoids**.

ii) Triterpenoids:

The bitterness of neem is due to the occurrence of **limonoids** which are the**tetranortripenoids** based on apo-euphal skeleton. The term limonoid is derived fromlimonin, the first tetranortripenoid obtained from citrus bitter principle in 1841;thestructure of which could be established only 1960. Out of 300 limonoids known todayabout 1/3 is accounted by neem(*Azadirachta indica*) and chinaberry (*Melia azedarach*)alone(Kumar*et al.,* 1996).

Neem bitter property can be conveniently classified under 8 groups:

Protomeliacins, limonoids with a modified side chain, azadirone and its derivative, gedunin and its derivatives, vilasinin type compounds, C -secomeliacins – nimbin, salanin and azadirachtin (Sundaram, 1996).

iii) Protomeliacins:

The triterpenes containing C8 side chains C- 17 are supposed to be bio-genetic precursorsof limonoids and hence known as protolimonoids or protomeliacins (Govindachari *et al.,* 1996).

Meliantriolwas the first triterpenyl alcohol, isolated from both neem oil andfresh fruits of *Melia azedarach* and shown to exhibit marked feeding inhibition againstDesert locusts (Butterworth and Morgan, 1968).

Siddiqui and his co-workers have added other protomeliacins nimbocinone,nimolinone and kulactone etc. Nimbocinonehas been isolated from neem leaveswhile most of the other constituents from fruit coats and whole fruits (Siddiqui *et al.,* 1975).

iv) Limonoids with intact four rings and γ-hydroxybutenolide side chain:

The presence of a γ-hydroxybutenolide side chain in place of the furan ring is thecharacteristics of this group of compound (Ley *et al.,* 1989). Two isomeric constituents, nimocinolides and isonimocinolide have been isolated from neem leaves where asisonimolicinolide from fresh fruits (Ley *et al.,* 1989). Nimocinolides showed mild insect growthregulating properties (Ley *et al.,* 1989).

v) Gedunin and its derivatives:

This group consists of compounds wherein the D- ring has undergone oxidativeexpansion.

Gedunin and its deacetyl derivatives have been found in neem barkalso in addition to their co-occurrence in seed oil (Khalid *et al.,* 1989). Gedunin was shown to posses both antifungal and anti malarial property(Rao *et al.,* 1977, Khalid *et al.,* 1989).

vi) Azadirone and its natural analogues:

This group consists of limonoids in which all rings of the triterpenoid skeletonremain intact. Characteristics features of this group are presence of oxygen function at C3and C7 (Govindachari *et al.,* 1996).

Butterworth and Morgan (1968) isolated Azadirachtin A. White Nahanishi first identified thegroup.

vii) C-Secomeliacins

This is a large and important group containing the most complex compounds. There are 3 important sub-groups in this form-

(i) nimbin

(ii) salanin

(iii) azadirachtin

Correctly 22 members of nimbin and salanin group have been isolated fromneem (Ganguli, 2002).Salanin has anti-feedant and detorant properties (Govindachari *et al.*,1996).

The isolation of nimbin in 1942 marked the beginning of the chemistry of neemmeliacins. The procedure for structural elucidation of nimbin was done very critically, carefully andcumbersome because no technology was known at that time. This particular chemistrywas done by 4 chlrol of chemistry (Ganguli, 2002).

II. Non-isoprenoid constituents:

i) (Poly) phenolics

a. Flavonoids: The neem leaves were reported to contain two flavonoids, quercetin andIsorhamnetin. The flowers were to contain kaempferol, myricetin and quercetin (Galeotti *et al.,* 2008). Theoccurrence of a new isoprenylatedflavanone, nimbaflavanone (8,3'-di-isoprenyl-5,7-dihydroxy-4'-methoxyflavanone) in leaves is also reported (Garg and Bhakani, 1984).

b. Flavonolglycosides*:* The occurrence of glycosides of kaempferol and quercetin inflowers and leaves and that of myricetin in leaves is reported. They are quercetin-3-galactoside, kaempferol-3-glucoside and myricetin 3'-L-arabinose (Aktar and Sengupta, 2008).

c. Dihydrochalcone: From the Aqueous fraction of the fruits, nimbochalcin, adihydrochalcone derivative was isolated (Leong*et al.*, 1998).

d. Tannis*:* Condensed tannis to the extent of 15% have been reported to occur in bark.

Aqueous extract of bark contained gallic acid, (+) gallocatechin, (-) epicatechin, (+)catechin have also been shown to inhibit the generation of chemiluminescence by activated humanpolymorphonuclear leukocytes (Vander Nat *et al.*, 1991).

e. Coumarin: There is only one report on the occurrence of a coumarin, scopoletin (7-hydroxy-6-methoxy coumarin) in leaves (Aktar and Sengupta, 2008).

ii) Carbohydrates and Proteins

The proteins are linked very tightly to the polysaccharides, whichconstitute the major components. Smaller gum components such as D-glucose, D-glucuronic acid, Larabinose, L-fucose mannose, xylose etc have beenidentified after drastic degradation of the complex (Kou1 *et al.*,1990, Sundaram,1996). The amino acid composition ofthe gum was also investigated and it has been found the most abundant was aspartic acid.Among others serine and threonine have been reported(Kou1 *et al.*,1990, Sundaram,1996).

a. *Polysaccharides Gla and Glb*: Gla is composed of a repeating unit consisting of onemolecule of α-L-arabinose and five molecule of α-D-glucose. The arabinose is linked(1-6) to one of the glucose molecules which are mutually linked (1-4). Glb is abranched arabinofucoglucan containing a main chain of (1-4) α -D-glucose

moleculessubstituted in the 6 position with side chains of α-L-arabinose molecules and in the 4position with 3-O-substituted fucose molecules (Fujiwara *et al.,* 1982).

b. *Polysaccharides GIIa and GIIIa*: Have significant anti-inflammatory effect on carrageenin – induced oedema in mice (Fujiwara *et al.,* 1984).

c. *Polysaccharides GIIIDO'2 Ia and GIIIDO'2IIa*: GIIIDO'2 Ia is a branchedfucogalactoglucoarabian containing a main chain of (1-5)-linked α -L-arabinose moleculesand (1-4) linked α -D-glucose molecules (Fujiwara *et al.,* 1982).

Two functionally different immunomodulators, one methanol insoluble, high molecularweight (10kD) saccharide-containing fraction and the other, methanol-soluble, lowmolecular weight (10kD) fraction were isolated from aqueous bark extract.

iii) Sulphurous Compounds

A number of cyclic tri- and tetra sulfides are obtainable from the steam distillate of thefresh matured leaves by GC-MS analysis. Several di- and trisulphides have also beenidentified by capillary GC-MS analysis of the headspace volatiles from crushed seeds.Di-n-propyl disulphide was shown to be the major compound (Pant *et al.,*1986).

Other chemical compounds include nimbocetin, a substituted aromatic easter from the fruits, methylgrevillate, margosinone, margosinolone (two new polyacetate derivative from the stembark) etc.

2.1.4 Importance of Neem to the Environment

Neem is known to increase soil fertility and water holding capacity. Thus large scale plantation of neem trees help to combat desertification, deforestation, soil erosion and to reduce excessive global temperature (Sateesh, 1998). Neem has high rate of photosynthesis and liberates more oxygen than many other plant species, thus purifying the atmosphere (Nigam *et al.,* 1994; Randhawa and Parmar 1993).

About 50,000 neem trees were planted to provide shades for Muslim pilgrims during Hajj over 10 kms on the plains of Arafat leading to marked impact on the areas microclimate, microflora, microfauna and sand soil properties. The plantation when full-grown is expected to provide shade to about two million pilgrims (Ahmed *et al.,* 1989; Anonymous, 2006).

Neem is useful as wind breaks in areas of low rainfall and high wind speed (Anonymous, 2006). 20% increase in grain yield was observed in millet crops protected by double rows of neem trees providing 500 km of wind break in the Maijia valley of Niger. Large scale planting of neem has been initiated in the Kwimba afforestation scheme in Tanzania. Neem plantations have been used for halting the spread of Sahara desert in the countries from Somalia to Mauritania (Anonymous, 2006).

A mature neem tree yield about 10-100 tons of dried biomass/ha (plate.1), comprised of leaves (50%) fruits and wood (25% each). Neem wood is durable and termite resistant

and thus used in making poles for house construction, furniture etc. In rural India, neem is a good source of firewood and fuel. Its charcoal has high calorific value (Anonymous, 2006). Neem has the ability to resprout after cutting and to re-grow its canopy after pollarding. Thus it is highly suited for pole production (Anonymous, 1992).

Neem products have water purifying activity. Neem leaf powder could be used as biosorbent for the removal of dyes like Congo red from water (Bhattacharyya and Sharma, 2004).

2.1.5 Veterinary Importance of Neem

Modern societies revert to nature for remedies and neem tree provides a promising means (Anonymous, 2006). In India, neem has been used for centuries to provide health cover to live stock in various forms. It has also been very widely used as animal feed. Neem extracts having antiulcer, antibacterial, antiviral properties are used successfully to treat cases of stomach worms, ulcers, cutaneous diseases, intestinal helminthiasis (Rao *et al.,* 1969, 1977, 1986; Srinivasan *et al.*, 2001; Baswa *et al.*, 2001). All parts of the plant(gum, bark, leaves, and seeds) are used to treat animals. Neem leaves have been mainly used as antiviral agents against vaccinia, variola, foulfox and Newcastle disease viruses (Rao *et al.,* 1969). The hot infusion of leaves is also used to treat swollen glands, bruises and sprains. Bark is effective against cutaneous diseases(Baladrin *et al.*, 1988). Seed and

kernel oil are used as antiseptic, antifungal and antibacterial agents (Rao *et al.,* 1977, 1986; Srinivasan *et al.,* 2001; Baswa *et al.,* 2001). Neem oil have been reported to have antihyperglycaemic effects (Anonymous, 2006). Alcohol and aqueous extracts of flowers of neem exhibits lethal effect against cattle filarial parasite *Setaria cervi (*Mishra *et al.,* 2005).

Livestock insects such as maggots, horn flies, blow flies and biting flies are controlled traditionally using neem (Anonymous, 2006). Neem leaves contain appreciable amount of proteins, minerals, carotene and adequate amount of trace minerals except zinc. They also have appreciable amount of digestible crude protein (DCP) and total digestible nutrients (TDN) (Koul *et al.,* 1990). Thus cattle, buffaloes, goat, sheep, camel are fed with neem leaves. Neem oil which is rich in long chain fatty acid is used in poultry feed (Anonymous, 2006). De-oiled neem seed cake is rich in essential amino acids, crude proteins, fibre contents, sulphur and nitrogen. The processed cake has good appetizer and wormicidal activity and can be used as an excellent poultry feed (Anonymous, 2006).

Aflatoxicosis caused by *Aspergillus flavus* which originates from contaminated poultry feed is prevented using neem leaves (Anonymous, 2006), because the leaf extract inhibits the production of aflatoxin by *Aspergillus parasiticus* (Allameh *et al.,* 2002) and patulin production by *Penicillium expansum* (Mossoni *et al.,*2004).

Plate 1: A matured neem tree showing green leaves and branches

2.1.6 Medical Importanceof Neem

Indians were among the first to be aware of medicinal properties of neem (Anonymous, 2006). Traditionally, many disorders like inflammation, infections, fever, skin diseases, dental disorders and others have been treated with different parts of neem such as leaves, flowers, seeds, fruits, roots and bark. Neem leaf exhibits a wide range of pharmacological activities viz., anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic, anticarcinogenic and immunomodulatory (Subapriya and Nagini, 2005).

Ayurvedic literature lists various medicinal uses of neem. It describes neem bark to be cool, bitter, astringent, acrid and refrigerant and useful in the treatment of tiredness, cough, fever, loss of appetite and worm infestation. The bark is reported to heal wounds and vitiate conditions of kapha, vomiting, skin disease excessive thirst and diabetes(Baladrin *et al.,* 1988). Neem leaves have also been reported to be beneficial for eye disorders and insect poison and to treat vatic disorder. It is reported to be antileprotic. Neem fruits are bitter, purgative, and antihelminthic (Anonymous, 2006).

Neem has been known in India as‘sarvaroga nivarini’ in Sanskrit, meaning ‘the curer of all ailments’. In rural India, delivery chambers are fumigated with burning bark of neem, dried margosa leaves are burnt to repel mosquitoes.Several viral diseases are treated with neem. In India, neem leaf paste has also been used to treat small pox, chicken pox and

warts. Neem twigs serve as toothbrushes in rural India and Africa (Anonymous, 2006).Dental gel containing neem leaf extract reduces the oral plaque index and bacteria count(Pai*et al.*, 2004).

Neem oil treated mosquito nets and mosquito-repellent tablet are now available in the north-east India. Gedunin(aliminoid) obtained from neem has activity similar to quinine against malaria pathogens(Anonymous, 2006).

The neem liminoids (Azadirachtin, salannin, deacetylgedunin) exhibited high larvicidal, pupicidal and antiovipositional bioactivity against malaria vector-*Anopheles stephensi*(Nathan*et al.,* 2005). Good antioxidant activity have been observed with neem leaf aqueous extract, flower and stem bark ethanolic extracts (Sithisarn *et al.*, 2005).

The ethanolic leaf extract of neem have caused cell death of prostate cancer cells(PC-3) by inducing apoptosis(Kumar *et al.*, 2006).

Neem leaf extracts are alsoantimutagenic. The extract exhibit strong antimutagenic activity in *China punctatus*, a fresh water fish model (Farah *et al.*, 2006) while aqueous extract of neem root and leaves reduce blood sugar level in rats exhibiting antidiabetic activity (Halim, 2003).

The bark extract have been shown to completely heal duodenal ulcers when administered at the dose of 30-60mg twice daily for 10weeks(Bandyopadhyay *et al.*, 2004).

Enhancement of antibody production and cellular mediated response by neem components helps in the treatment of AIDS(Anonymous, 2006).

Neem seed oil showed bactericidal (Srinivasan *et al.*, 2001, Baswa *et al.*, 2001) and contraceptive (Anonymous, 1992, Upadhyay *et al.*, 1994) activities.

A vaginal contraceptive, NIM76 was developed from neem oil having antimicrobial activity against *Escherichia coli, klebsiella pneumonia* and *Candida albicans*(SaiRam *et al.*, 2000).

2.1.7 Industrial Uses of Neem

Several industries including pharmaceuticals, cosmetic, disinfectants, rubber, bio-pesticide and textile industries use neem oil (Jattan *et al.*, 1995). Many commercial preparations are currently available (Anonymous, 1993, Khanna, 1992).In India, neem is highly exploited by many ayurvedic drug industries.Neem oil and powdered neem leaves are employed in various cosmetic preparations such as face creams, nail polish, nail oils, shampoos, conditioners(Anonymous, 2006, Jattan *et al.*, 1995, Anonymous, 1992).

Heukelback *et al.*, 2006 observed that a shampoo, based on seed extract of neem was highly more effective than permethrin-based product against head lice under *in vitro* conditions.

Neem cake; a by product of neem oil industry is used as livestock feed, fertilizer and natural pesticides. Neem oil is commonly used in soap and toothpaste production**(**Koul*et al.*, 1990).Neem is a source of many oral- hygiene preparations and dental care products. Neem bark yields gum and tannins which are used in tanning, dyeing etc. Neem seed pulp is used as a rich source of carbohydrate in fermentation industries and for methane gas production. Cultivation of neem, collection of seed and processing of neem products provides employment and income generation opportunities for the poor household, especially in rural women (Koul*et al.*, 1990,Anonymous,2006). India comes first in neem production and about 540, 000 tons of seeds are produced annually yielding 107, 000 tons of neem oil and 425, 000 tons of neem cake(Anonymous,1993).

The amount of azadirachtin available is estimated to be about 1600 tons per annum, providing enormous amount of raw materials for pesticides industry. In the product sector, annual estimated turnover is about Rs. 1000-1200 crores.

2.2 The parasite: *Haemonchus contortus*

2.2.1 Geographical Distribution

Haemonchus contortus has been found in Asia (Indonesia, India), Netherlands, Europe (Russia, Italy), South America (Brazil), Africa, as well as in the United States. This parasite has adapted to conditions ranging from tropical areas to cold, mountainous regions. (Dorny *et al.*, 1996; Eckert and Hertzberg, 1994; Newton, 1995)

2.2.2 Habitat

Although this parasitic nematode has a great habitat range, it is more prevalent in sub-temperate and temperate regions under warm and wet conditions (Fleming, 2006).Being originally a tropical parasite, it has been disseminated around the world by livestock movement and can now be found as far north as the Arctic Circle (White and Newton, 2001). *Haemonchus contortus* inhabit the abomasum (fourth stomach) of ruminants. It has been found in humans in Brazil and Australia. (Roberts and Janovy, 2000).

2.2.3 Morphology

Haemonchus contortus is cylindrically shaped, tapered at both ends, and has a complete digestive system. It has a cuticle with three main outer layers made of collagen and other compounds, (Roberts and Janovy, 2000). The outer layers are non-cellular and are secreted by the epidermis. The cuticle layer protects the nematode so it can invade the digestive tracts of animals. The worms molts four times, the first two before hatching, and the last two before their adult stage.

Since it is a blood-sucker, *Haemonchus contortus* generally has a reddish appearance. The white ovaries that wind around the blood filled intestine, gives the nickname "barber's pole", when referring to the females. The females have a length ranging from 18-30 mm, while the males are shorter, ranging from 10-20 mm. The male's distinct feature is its well-developed copulatory bursa, containing an asymmetrical dorsal lobe and y-shaped dorsal ray (Roberts and Janovy, 2000).

As a Secernentea, *Haemonchus contortus* has a specialized tubular excretory system with three canals. The canals are arranged to form an "H" (Barnes, 1987, Roberts and Janovy, 2000). The oocyte is yellowish in color. The egg is approximately 70–85 μm long by 44 μm wide, and the early stages of cleavage contain between 16 and 32 cells.

2.2.4 Life Cycle and Development

The female deposits 5000-10,000 eggs in the abomasum per day, which eventually will pass out with feaces of the host. In the first stage, the juveniles hatch from the eggs. During the first and second juvenile stages, they will feed on bacteria in the manure. In the third juvenile stage that is the L3 infective stage, the ruminant becomes infected when eating contaminated forage. Prior to further development, exsheathment, which is sheddding of the cuticle, takes place in the host's gut. *Haemonchus contortus* exsheath when it is stimulated by high CO_2 and elevated temperatures in the host. After exsheathment, the worm will pass into the abomasum where it will burrow into the mucosa. Here, it will molt and in the fourth stage finds its way back to the lumen of the abomasum, feeds and undergoes a final molt before reaching adulthood. (Chappell, 1979; Fetterer and Rhoads, 1996; Roberts and Janovy, 2000; White and Newton, 2001)

2.2.5 Reproduction

Females may produce a pheromone to attract males. The male coils around a female with his curved area over the female genital pore. The gubernaculum, made of cuticle tissue, guides spicules which extend through the cloaca and anus. Males use spicules to hold the female during copulation. Nematode sperm are amoeboid-like and lack flagella. (Barnes, 1987; Roberts and Janovy, 2000)

2.2.6 Behaviour

The behavior of parasitic nematodes has evolved to have certain adaptations. For instance, during its infective stage, the third juvenile stage of*Haemonchus contortus*migrates onto grass blades to make it more optimal for host ingestion. While adapting to extreme temperatures and conditions, the juvenile parasite will prefer to lay eggs in the "adapted" conditions even when given less harsh conditions. Furthermore, moist environments are optimal for laying eggs; the juvenile prefers to reproduce in dry conditions when given the option between the two. (Li *et al.*, 1999).

2.2.7 Communication and Perception

Nematodes within the Secernentea have phasmids, which are unicellular glands. Phasmids likely function as chemoreceptors. Females may produce pheromones to attract males.

Nematodes in general have papillae, setae and amphids as the main sense organs. Setae detect motion (mechanoreceptors), while amphids detect chemicals (chemoreceptors) (Roberts and Janovy, 2000).

2.2.8 Food Habits

During the first and second juvenile stages, *Haemonchus contortus* feeds on bacteria in manure. In the later stages, *H. contortus* parasitizes the abomasum, the "true stomach", in cattle, goats, other wild ruminants, but mainly sheep. In the abomasum, it feeds on blood using a single, dorsal tooth to cut into the host tissue.

Pharyngeal glands and intestinal epithelium produce digestive enzymes to feed on the hosts' body fluids. Extracellular digestion begins within the lumen and is finished intracellularly. (Barnes, 1987; Fetterer and Rhoads, 1996; Newton and Munn, 1999; Roberts and Janovy, 2000)

2.2.9 Pathology/ Diseases

Haemonchus contortus suck blood and cause anaemia in the animals affected. They cause severe to fatal, rapidly developing disease or longer term anaemia causing production loss.

Signs of *H. contortus* infestation includes: diarrhoea; dehydration; unthrift appearance, rough hair coat, depresssion, low energy, lethargy, and uncoordinated movement; significantly reduced growth and reproductive performance; fluid accumulation in sub-

mandibular tissues (bottle jaw), abdomen, thoracic cavity, and gut wall; blood loss, white mucous membranes, anaemia/PCV (Smeal, 1995, Love and Hutchinson 2003).

Effects

1. *Haemonchus* suck blood from the lining of the stomach, causing anaemia.
2. Sheep with heavy infections of *Haemonchus* lack stamina, have pale gums and conjunctiva, and may also have bottle-jaw or constipation. Sheep with lighter burdens have a gradual onset of weight loss and loss of colour in the gums and conjunctiva
3. If present in large numbers, *Haemochus* can kill sheep. In these animals, large red masses of worms are clearly visible in the stomach. The stomach contents are often brown because of bleeding from the stomach lining and the lining has pin-point blood spots on it. The blood of infected sheep appears watery due to anaemia.
4. In sheep with lighter infections, worms are present in the stomach, but the lining of the stomach looks normal.

2.2.10 Diagnosis

In diagnosing helminthiasis, the three pillars of veterinary diagnosis are: history, clinical signs and gross pathology, and laboratory aids. Also, knowledge of the local nuances of the parasite epidemiology and control is invaluable.

Feacal worm egg counts (FECs) (preferably with speciation by way of larval culture and differentiation), and total worm counts are the tests most commonly employed in the diagnosis of helminth infections in ruminants. However FEC does not always correlate well with the number of adult worms present, particularly in cattle aged 9-12 months old. 'Diagnostic drenching' may be a useful tool in such cases. FECs may also be low or zero in the presence of large numbers of immature worms. Necropsy is the most direct method to diagnose gastrointestinal (GI) parasitism such as *Haemonchus* (Smeal, 1995; Love and Hutchinson 2003).

2.2.11 Economic Importance for Humans

The major problem lies within the agricultural industry. These parasites cause great economic losses in domestic animals, specifically sheep, cattle and goat. Because *Haemonchus contortus* is a blood sucker, it can induce anemia and oedema. Also, the hemolytic proteins that the parasite releases can lead to other intestinal disturbances and a heavily infected host often dies.

Haemonchus contortus is known to adapt well to harsh conditions making it difficult to eliminate the parasite(Jacquiet et al., 1998; White and Newton, 2001) leading to failure of treatment and control.

2.2.12 Control

Scientists have been researching ways to manage this parasitic worm and minimize cost production in herds. Management practices must be based upon breaking or interrupting the life cycle of the worm by:

• Use of chemical antihelminthics commercially available

• Improved herd and pasture management practices

• Use of plants with antihelminthic properties

2.2.13 Use of Commercial/ Synthetic Antihelminthics

The use of antihelminthics is limited by their high cost, uncertain availability and poor quality (Monterio *et al.*, 1998). Antihelminthics can be classified as follows:

2.2.13.1 The Broad Spectrum Antihelminthics:

The broad-spectrum antihelminthics, which remove parasites in different stages of development within the host species, are the cornerstone of parasite control in gastro intestinal nematode infection. The major classes of synthetic antihelminthics used for gastro intestinal nematode parasites of ruminant livestock are:

I. The benzimidazole/probenzimidazoles group. The mode of action of this group is by interference with polymerization of microtubules (Harder, 2002). These drugs bind to the protein tubulin of the parasite, thereby causing death by starvation (Roos, 1997).

II. The tetrahydropyrimidines group (levamisole/pyrantel-morantel). These Drugs affect acetylcholine neuro-transmission by interacting with nicotinic acetylcholine receptors (Roos, 1997: Harder, 2002).

III. The macrocyclic lactones or ivermectin/milbemycin group. These groups are thought to interact with chloride channels on the gamma-aminobutyric acid (GABA) receptor complexes of helminths, and also inhibit pharyngeal pumping (and hence feeding), motility and fecundity in susceptible nematodes, resulting in paralysis and ultimately, elimination from the host (Harder, 2002; Yates *et al*., 2003).

2.2.13.2 The Narrow Spectrum Antihelminthics:

These are group of compounds which have activity against fewer species of parasites and/or lack high levels of efficacy against some or all stages of the parasites (Bowman *et al*., 2003). Examples of these antihelminthics include napthalopos, salicylanilide and substituted phenols (closantel, oxyclozanide and nitroxynil), and tricalbendazole (Bowman *et al*., 2003).

2.2.14 Improved Herd, Pasture and Management Practices

Haemonchus contortus/ barber pole worms can be controlled by:

2.2.14.1 Feeding Practices

This should be done by keeping stocking rates low, provision of clean water and minerals, use of gravel or concrete in the feedlot area to break the worm life cycle and to prevent re-infestation, provision of high-quality hay for goats raised in areas where forage is not available year-round or when the pasture conditions are poor and lastly the hay should be kept off of the ground to avoid contamination by faeces (Van Wyk *et al.*, 1997, Waller *et al.*, 2001).

2.2.14.2 Pasture Maintenance

Goats love to browse and prefer shrubs and forages to grass. If goats consume forages at least 6 inches tall, infestation from *H. contortus* is reduced.

The use of pasture for hay cutting after grazing helps to break the worm life cycle and prevent re-infestation. Direct sunlight during the summer months or during freezes in the winter also helps decrease the population of larvae that remain in the soil(Jacquiet *et al.*, 1998, Burke, 2005).

Alternating the pasture with a short cycle crop such as culture alfalfa breaks the worm's life cycle and decreases larvae population in the pasture thereby preventing re-infestation.

2.2.14.3. Treatment of Ewe/ Doe

Well-nourished animals are better able to resist*H. contortus* infestation. Animals with good nutrition at late pregnancy have an increased immune response to parasites. The pregnancy hormone progesterone and the lactate hormone prolactin have been shown to reduce the ability of the ewe/doe to fight worm infestation.

Progesterone suppresses the ewe or doe’s immune system. Animals that are lactating and raising kids are more susceptible to worms. Monitor the herd closely from kidding to weaning.

Goat genetics isalso an important component that will determine an animal's ability to resist *H.contortus* infestation because resistance to worm infestation is heritable. The ewe

or doe's ability to withstand infestation is defined as Dry lot to interrupt worm life cycle (Newton, 1995, Van Wyk *et al.,* 1997)**.**

2.2.14.3.1. Famacha©System(Clinical Anaemia Evaluation) - A Management Tool for the Control of *Haemonchus Contortus*

This is a system that uses visual observation of the redness of the interior of the eyelid using a specially developed eye color chart to determine the degree of infection due to *Haemonchus contortus.*

The name FAMACHA© was coined to describe the system evolved for treating only those animals unable to cope with current *Haemonchus contortus* challenge on pasture, by using clinical anaemia as the determinant. It is an acronym derived from the name of the originator of the idea, Dr Faffa Malan (**FA**ffa **MA**lan**CHA**rt) (Bath *et al.,* 1996, Van Wyk and Bath, 2002). It is common knowledge that during the course of fatal haemonchosis the colour of the conjunctivae of sheep changes from the deep red of healthy sheep, through shades of pink to practically white, as a result of a progressively worsening anaemia.

Based on this chart, only goats with anaemia should be treated. This practice will prevent the overuse of antihelminthics and consequently minimize chances of parasite resistance to antihelminthics. The test has an effectiveness of 90 percent compared to the 20-30 percent effectiveness of overused antihelminthics.

The extent to which these changes relate to a range of haematocrit (Ht) values (chosen as the "gold standard" of anaemia) (Jain, 1986) was, until recently, still undetermined. The feasibility of grading the degree of anaemia clinically in conjunctival mucous membranes was investigated by both photographing the mucous membranes and determining the Ht values of sheep which ranged from very healthy to extremely anaemic (Bath *et al.,* 1996, Malan and Van Wyk, 1992, Malan *et al.,* 2001, Van Wyk *et al.,* 1997).

The FAMACHA system is a low-cost tool that can greatly influence management practices in goats.

FAMACHA also provides producers with a tool for genetic selection. Using FAMACHA, producers can identify animals that are more susceptible to hosting the worm and can cull these goats from the herd. In turn, producers will be able to identify animals with high resistance and resilience, which seem to be inherited traits in goats.

2.2.15 Use of Plants with Antihelminthic Properties

Researchers are exploring the use of plants to control *Haemonchus contortus*. Forages, such as clover, vetches chicory, and *Sericea lespedeza,* contain condensed tannins. Condensed tannins reduce the number of stomach worms and egg production (Niezen *et al*., 1998; Kahn and Diaz-Hernandez, 2000; Athanasiadou *et al*., 2001; Waller *et al*., 2001). Feeding *Sericea lespedeza* hay to goats can reduce fecal eggs counts by 80 percent and create a higher packed cell volume (Niezen *et al.,* 1998, Waller *et al.,* 2001).

CHAPTER THREE

3.0 Materials and Methods

3.1.0 Collection of Plant Materials

Different parts (leaf, stem bark and root) of *A. indica*(neem) were collected in Zaria. The plant was identified, confirmed and assigned voucher number 900151 in the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University,Zaria.

3.2.0 Preparation of the Plant Parts

Different parts (leaf, stem bark and root) of *A. indica*(neem) were air dried at ambient temperature after which each was pulverized separately using pistle and mortar and later sieved, using British pharmacopeia graded sieve No. 44. 500g of each plant part was stored concealed at room temperature until required for use (Nwude *et al*., 1983).

3.3.0 Methanolic Extraction

The solvent extraction technique described by Nwude *et al*. (1983) and Sofowora (1982) was performed. Five hundred grams (500g) of each plant parts collected was defatted in 2.5 litres absolute methanol at room temperature for eight hours to obtain the methanolic extract. The extract was filtered and the filtrate evaporated to dryness in a rotary evaporator (Buchi, Switzerland) at 50^0C under reduced pressure. The extracts were collected and concealed in aluminium foil paper and stored in a refrigerator at 4^0C until required.

3.4.0 Phytochemical Screening

Standard screening tests were carried out on the methanolic extract to determine the phytochemicals present using the standard procedure of Trease and Evans (1983) and Sofowora (1982).

3.4.1: Test for Carbohydrate

- **Molisch's test:** Two to three drops of Molisch's reagent was added to 0.5g of extract in a test tube and 2 ml of concentrated sulfuric acid was allowed to run

down the side of the test tube to form a lower purple to violet colour at the interface. This indicates the presence of carbohydrate (Trease and Evans, 1983).

Confirmatory Test

- **Fehling's Test:** To 2 ml of extract, 5 ml of a mixture of Fehlings solution A and B in the ratio of 1:1 were added and the mixture boiled for two to three minutes. A brick red precipitate indicates the presence of free reducing sugar (Trease and Evans, 1983).

3.4.2 Test for Glycoside

5 ml of concentrated sulphuric acid was added to 5mlof extract and boiled for 15mins. This was then cooled and neutralized with 20% KOH and was divided into two portions, another part of extract was dissolved in distilled water which was used as a control.

- **Fehling's Solution Test:** 1:1 Fehling's solution A and B was added to 2ml of extract and boiled for two to three minutes, a brick red precipitate indicates the glycone portion as a result of hydrolysis of glycoside.

Confirmatory Test

- **Ferric Chloride Test:** Three drops of ferric chloride solution was added to 5ml of the extract. Green to black precipitate indicates phenolic aglycone as a result of glycoside (Trease and Evans, 1983).

3.4.3 Test for Free Anthraquinones Derivatives

Test for Free Anthraquinones (Borntragers' test): Two grams of the extract was mixed with 10 ml of benzene, shaken vigorously and filtered. 5ml of 10% of ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet colour indicates the presence of free anthraquinones (Trease and Evans, 1983).

Confirmatory Test

- **Test for combined anthracene (Modified Borntragers' test):** Two grams of extract was boiled with 5 ml of 10% hydrochloric acid for three minutes; this will hydrolyze the glycosides to yield aglycones which are soluble in hot water only. The solution was filtered hot; the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was filtered off and shaken gently with half its

volume of 10% ammonia solution. A rose pink or a cherry colour indicates anthracene (Trease and Evans, 1983).

3.4.4 Test for Cardiac Glycosides

- **Kella- Killaini Test:** Two grams of extract was dissolved in glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45^{o}; 2 ml of concentrated sulpuric acid was added down the side, purple ring color indicates cardiac glycosides (Trease and Evans, 1983).

Confirmatory Test

- **Kadde Test:** 2 ml of 2% 3, 5-dinitrobenzoic acid in 95% alcohol was added to 0.5g of extract, the solution was made alkaline with 5% sodium hydroxide, an appearance of purple-blue colour indicates the presence of cardenolides in the ring (Trease and Evans 1983).
- **Salkowsk Test:**0.5g of extract was dissolved in 2 ml of chloroform, two to three drops of concentrated H_2SO_4 was added to form a lower layer. A reddish brown colour at interface indicates the presence of a steroidal ring (Trease and Evans, 1983).

3.4.5 Test for Saponins

- **Frothing Test:**Two grams of the extract was dissolved in 10ml of distilled water. This solution was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb formed for more than 30 minutes indicate saponin (Trease and Evans, 1983).

3.4.6 Test for Steroid and Triterpenes

Equal volume of acetic anhydride was added to 5ml of extract. Concentrated sulphuric acid (2ml) was added down side the tube, the colour change was observed immediately and later, pink or purple colour indicates the presence of tritepenes while blue or blue-green indicate steroids (Trease and Evans, 1983).

3.4.7 Test for Flavonoids

- **Shinoda Test:** 0.5g of extract was dissolved in 2 ml of 50 % methanol and heated in a bunsen burner. Metallic magnesium and five drops of concentrated hydrochloric acid was added into the mixture. A red or orange colour indicates the presence of flavanoic aglycones.

Confirmatory Test

- **Sodium Hydroxide Test:** Two to three drops of aqueous NaOH was added to 5ml of extract, a yellow colouration shows the presence of flavonoid (Trease and Evans, 1983).

3.4.8 Test for Tannins

- **Lead Sub-acetate Test:** Three dropsof lead-sub acetate solution was added to 5ml of the extract. A coloured precipitate indicates tannins.

Confirmatory Test

- **Ferric chloride:** Two grams of extract was dissolved in 10ml of distilled water, and then filtered. Two to three drops of ferric chloride solution was added to the filtrate. Formation of a blue-black precipitate indicates hydrolysable tannins and green precipitate indicates the presence of condensed tannin (Trease and Evans, 1983).

3.4.9 Test for Alkaloids

- **Meyers Test:** Two to three drops of Meyers reagent was added to 5ml of extract, rose red precipitate indicate the presence of alkaloids (Sofowora, 1982).

Confirmatory Test

- **Dragendoff's Test:** Two to three drops of this reagent was added to 5ml of extract, rose red precipitate indicate the presence of alkaloid.
- **Wagners Test:** A drop ofWagners reagent was added to 2ml of extract, whitish precipitate indicates alkaloids.

3.5.0 Quantitative Phytochemical Screening

3.5.1 Estimation of Total Flavonoid Content

The total flavonoid content was determined according to Eom *et al.* (2007). An aliquot of 0.5 ml of extract (1 mg/ml) was mixed with 0.1 ml of 10% Aluminum nitrate and 0.1 ml of potassium acetate (1 M). In the mixture, 4.3 ml of 80% methanol was added to make the total volume of 5 ml. The mixture was shaken vigorously and the absorbance was measured spectrophotometrically using thermo electron ~ visionpro software V3.00 at 415 nm within 10 minutes and compared with standard.

3.5.2 Estimation of Total Tannin Content

Total tannin content was determined by Van-Burden and Robinson (1981) method: 500mg of the plant parts was weighed into a 50ml plastic bottle. 50ml of distilled water was added and shaken for one hour in a mechanical shaker. This was filtered into a 50ml volumetric flask and made up to the mark. Then 5ml of the filtrate was pipetted out into a test tube and mixed with 2ml of 0.1M $FeCl_3$ in 0.1N HCl and 0.008 M Potassium ferrocyanide. The absorbance was measured at 415nm within 10 minutes and compared with standard.

3.5.3 Estimation of Total Saponin Content

Total saponin content was determined by Obadoni and Ochuko (2001) method. Three samples 20g each of the plant parts were put in a conical flask and $100cm^3$ of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55^0C. The mixture was filtered and the residue re-extracted with another 200ml 20% ethanol. The combined extracts were reduced to 40ml over water bath at about 90^0C. The concentrate was transferred into a 250ml separatory funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60ml of n-butanol was added. The combined n-butanol extracts were washed twice with

10ml of 5% aqueous NaOH and heated in a waterbath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

3.5.4 Estimation of Total Alkaloid Content

10g of powder plant parts was weighed and mixed with 50ml of four volumes of solvent ether and one volume of alcohol (95 percent) shakened and allowed to stand for 10 minutes followed by addition of 1.5ml dilute ammonia solution mixed with 2ml of water and shaken frequently during one hour. The mixture was transferred to a small percolator plugged with cotton wool and, when the liquid cease to flow, it was packed firmly and the percolation was continued with 25ml of ether alcohol mixture and then with solvent 25ml ether until the extraction of the alkaloid was completely effected. The volume of the percolate was reduced to 20ml by distillation on a water bath transferred to a separator, washing the flask with three quantities each of 10ml of chloroform, and added to the separator followed by 20ml of N/2 Sulphuric acid and shaken. It was allowed to separate and the lower layer run off.

The extraction was continued with successive quantities, each of 10ml of the mixture of 3 volume of N/10 Sulphuric acid and 1 volume of alcohol 95% until complete extraction of the alkaloid was effected. The mixed acid solution was washed with 10, 5, and 5ml of

chloroform and extracting each chloroform solution with the same 20ml of N/10 Sulphuric acid and rejecting the chloroform. The combined acid solution was neutralized with dilute ammonia and 5ml in excess, shaken with successive quantities each of 25ml of chloroform until extraction was completed. The chloroform solution was washed with the same 10ml of water and filtering into a flask through a plug of cotton wool previously moistened with chloroform. Most of the chloroform was distilled and the remainder of the solution transferred to a shallow open dish. The remainder of the chloroform was evaporated; the residue was heated in an oven at 100^0C for 15 minutes. The residue was dissolved in 2ml of chloroform, added 10ml of N/20 Sulphuric acid, warmed to remove the chloroform, cool and the excess acid was titrated with N/20 Sodium Hydroxide, using methyl red as indicator. Each ml of N/20 Sulphuric acid is equivalent to 0.01447g of alkaloid calculated as Hyoscyamine (Grainger, 1969).

3.6.0 LC50/EC Values Analysis

The lethal concentration LC50/EC values for the leaf, stem bark and root of *A. indica* were computed using the EPA Probit Analysis Programme after 48 hours of exposure to the various plantsextract (Lorke, 1983).

3.7.0 Procedure for Collection of Eggs.

The collection of *H. contortus* eggs for the *in vitro* studies was done using the modified McMaster method described by Sloss *et al*. (1994). Abomasums of goats were obtained from Zaria Abattoir and conveyed in clean polythene bags to the Helminthology Laboratory, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The abomasums were cut open and the abomasal content placed on a fluorescent picking board, the parasites were picked with picker and transferred to petric dishes containing water. Female adult *H. contortus* identity was confirmed using standard taxonomic features.

The parasites were crushed in mortar with pestle. 60 ml of water was added to the crushed worms, and filtered in a 100 mesh sieve (150µm).

3.8.0 Cultivation and Recovery of *Haemonchusconrortus* Larvae

Eggs of *H contortus* were cultured in culture plates and maintained at 27 ℃ in an incubator. The infective third stage larvae (L3) were recovered from 7-9 day old sterile feacal cultures using a modified Baermann Apparatus according to the method of Suleiman (2002).

Cultured plates were filled with tap water and inverted onto clean petri dishes. 10 ml of water was then placed around the bottle in the petri dishes. It was allowed to stand for at

least 5 hrs to allow the larvae migrate out of the culture materials in the inverted sample bottles. The water in the petri dishes were then transferred into clean 250 ml beakers and allowed to stand for one hour to allow the parasites settle at the bottom of the beaker. Thereafter, the water in the beaker was decanted in order to concentrate the parasites.

The larvae harvested were concentrated at 1000 rpm for 15 minutes. About 0.1 ml of the concentrated larvae was pipetted and spread onto a glass slide; a drop of Lugol iodine was applied to immobilize the L3 larvae. The slide was mounted on a microscope and the L3 observed and counted at x400 magnification. The process was repeated five times and the mean average numbers of larvae were recorded. Water was added where necessary, and the volume adjusted such that 0.1 ml of the solution contained about 120 infective larvae (L3).

3.9.0 *In-vitro* Larval Mortality Test

120 larvae in 0.1 ml in wells of microtitre plate and concentrationsof each of the extracts (leaf, stembark and root) at 0.1mg/ml, 1.0mg/ml, 10.0mg/ml, 100mg/ml, negative control (water) and positive control (Levamisole) were added to each of the wells with six replication each as shown in plate2.

After addition of the treatments, it was then viewed under the microscope once in 6 hours for a period of 48 hours and the number of mortality recorded.

Data was expressed as percentage mortality using the formula of Cavier (1973); N-n/N x 100

Where N=Total number of larvae in control wells.

n= number of mortalities.

3.10.0 Statistical Analysis

Analysis of variance - ANOVA (Factorial) was employed to test if there were significant differences between the different plant parts, the various concentrations and the time of exposure.

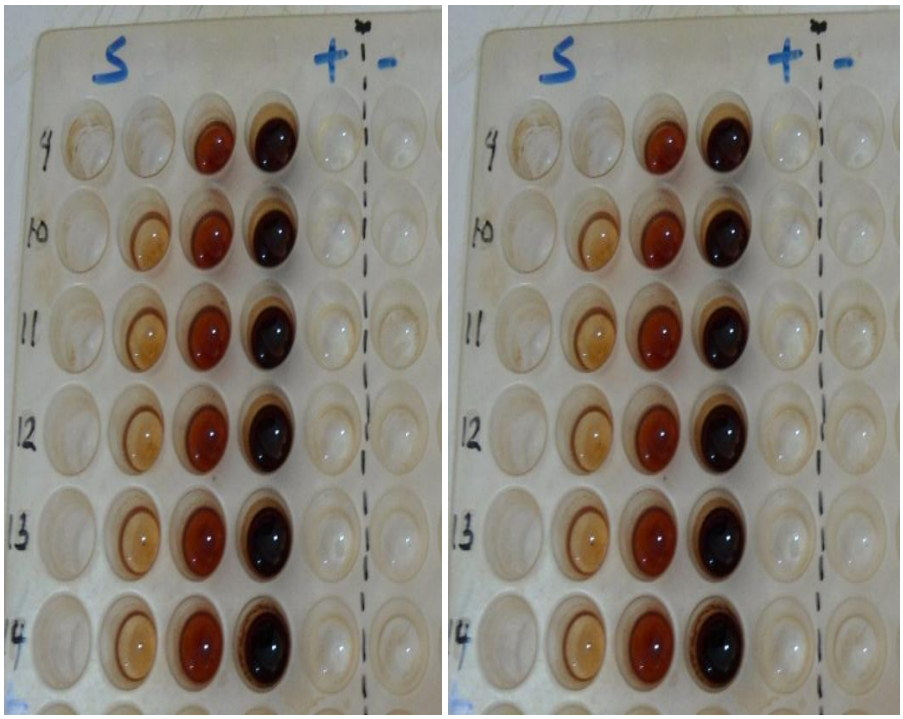

Plate 2. Microtitre plates with different concentrations and replications of the plant extracts

CHAPTER FOUR

4.0 Results

4.1.0 Phytochemical Constituents

The Phytochemical screening of the methanolic extracts of leaf, stembark and root of *A. indica* (neem)showed the presence of the following secondary metabolites: carbohydrate, cardiac glycosides, saponins, flavonoids, tannins and alkaloid; steroids were absent in stembark and root while leaf showed presence of steroids; triterpenes absent in leaf and presence in both stembark and root whereas anthraquinones derivatives were absent in all of the three plant part extracts (Table 1). The quantitative estimation of some phytochemical constituents of leaf, stem bark and root of *A. indica* (neem) revealed that saponins, flavonoids, tannins and alkaloids had the following respective percentages 0.75, 0.68, 0.67; 20.21, 27.50, 20.95; 4.95, 4.93, 4.15 and 5.25, 5.48, 5.48 (Fig.1).

4.2.0 LC50/EC Values Analysis

The lethal concentration (LC50/ EC) experimental analysis gave a value of 12.30mgml^{-1} for the leaf, 12.58mgml^{-1} for the stem bark and 15.84mgml^{-1} for the root extract with Log. X, linear equation, probits, mortalities and concentrations of the various plant partsagainst *Haemonchus contortus* (Table 2). The points at which the probit cut across the 50% is the interception with the corresponding x value whose antilog is the LC50 value (Fig. 2).

Table 1: Qualitative Analysis of the Phytochemicals of Leaf, Stembark and Root of *Azadirachta indica*

CONSTITUENTS	LEAF	STEMBARK	ROOT
Carbohydrates	+	+	+
Cardiac Glycosides	+	+	+
Anthraquinones Derrivatives	-	-	-
Saponin	+	+	+
Steroid	+	-	-
Triterpenes	-	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Alkaloids	+	+	+

Key (+) = presence

(-) = absence

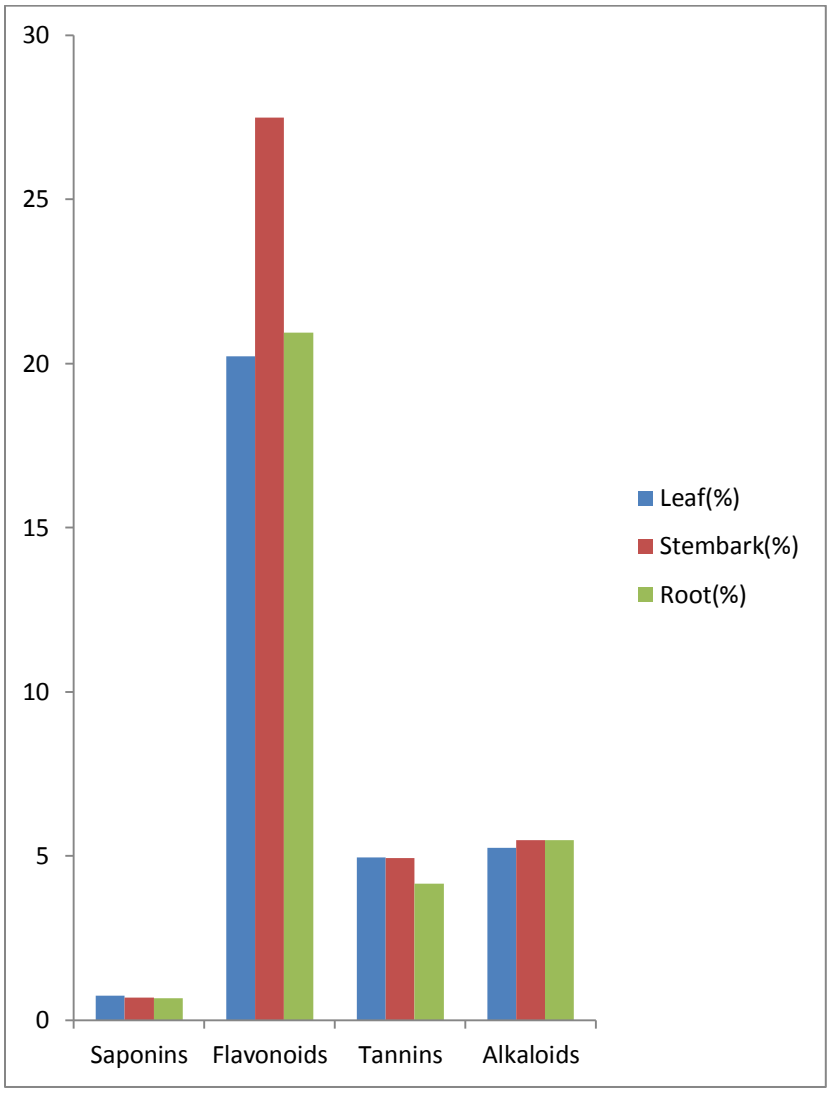

Fig.1: Quantitative Estimation (Per Gram) of Some Phytochemicals of Leaf, Stembark and Root of *Azadirachta indica*

Table 2. LC50 values, Log. X, linear equation, probits, mortalities and concentrations of leaf, stem bark and root of*Azadirachta indica* against *Haemonchus contortus*

	Mortalities			Probits			Linear Equation	Log. X	Antilog x (LC50)
Conc.	Leaf	Stembark	Root	Leaf	Stembark	Root			
0.1	11.67	11.67	10.83	3.82	3.82	3.77	Leaf y= 0.583x+4.361	1.09	12.3
1	25.83	25	24.58	4.36	4.33	4.33	Stem bark y= 0.576x+4.362	1.1	12.58
10	43.33	45.83	41.67	4.82	4.9	4.8			
100	73.33	70.83	70	5.61	5.55	5.52	Root y= 0.572x+ 4.319	1.2	15.84

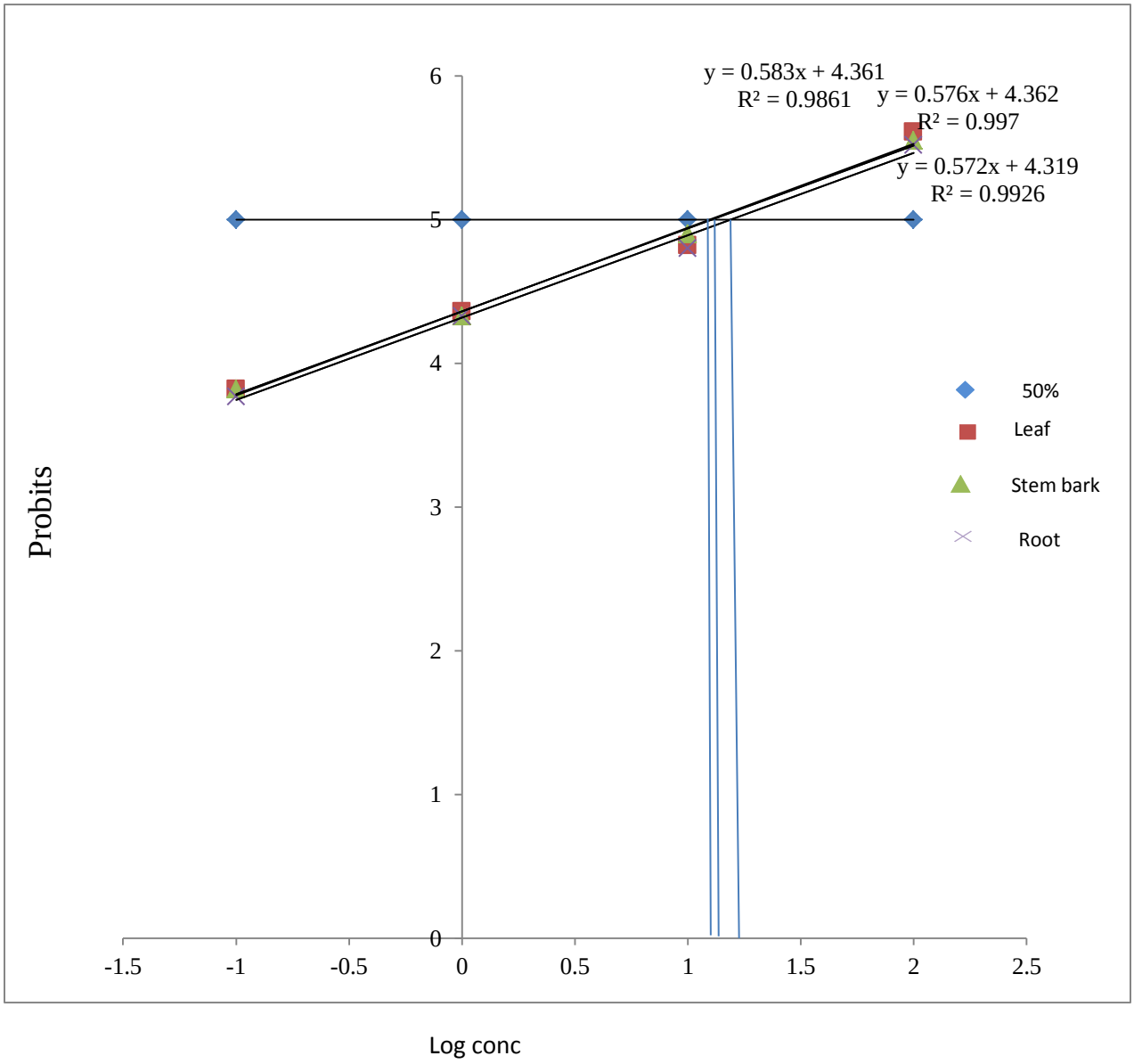

Fig. 2:Log doses and probits of leaf, stem bark and root methanolic extracts of *Azadirachta indica* against *Haemonchus contortus* with points of interception

4.3.0 *In-vitro* Larval Mortality Test

The *in vitro* nematocidal activity of methanolic extracts of leaf, stembark and root of *A. indica* (neem) showedthat the three extracts affected the motality of the parasite, the negative control was not effective as there were no mortalities or only one mortality recorded but the positive was the most effective. Mortalities increased with increase in concentration and with the time of exposure (Fig. 3, 4 and 5).

4.4.0 Analysis of Variance for the Total Data

Analysis of variance (ANOVA) summary for the total data showing level of significance for plant parts, time, concentration, plant part and time, plant part and concentration, time and concentration, plant part, time and concentration (Table.2).

The data showed highly significant differences between the plant parts, time of exposure, concentration of the extracts, the time of exposure and concentration and between the plant parts and concentration ($p=0.001$). There were no significant differences between the plant parts and the time of exposure ($p=0.92$) and between the plant parts, time of exposure and concentration ($p=0.99$), though mortality of the parasites increased with increase in time.

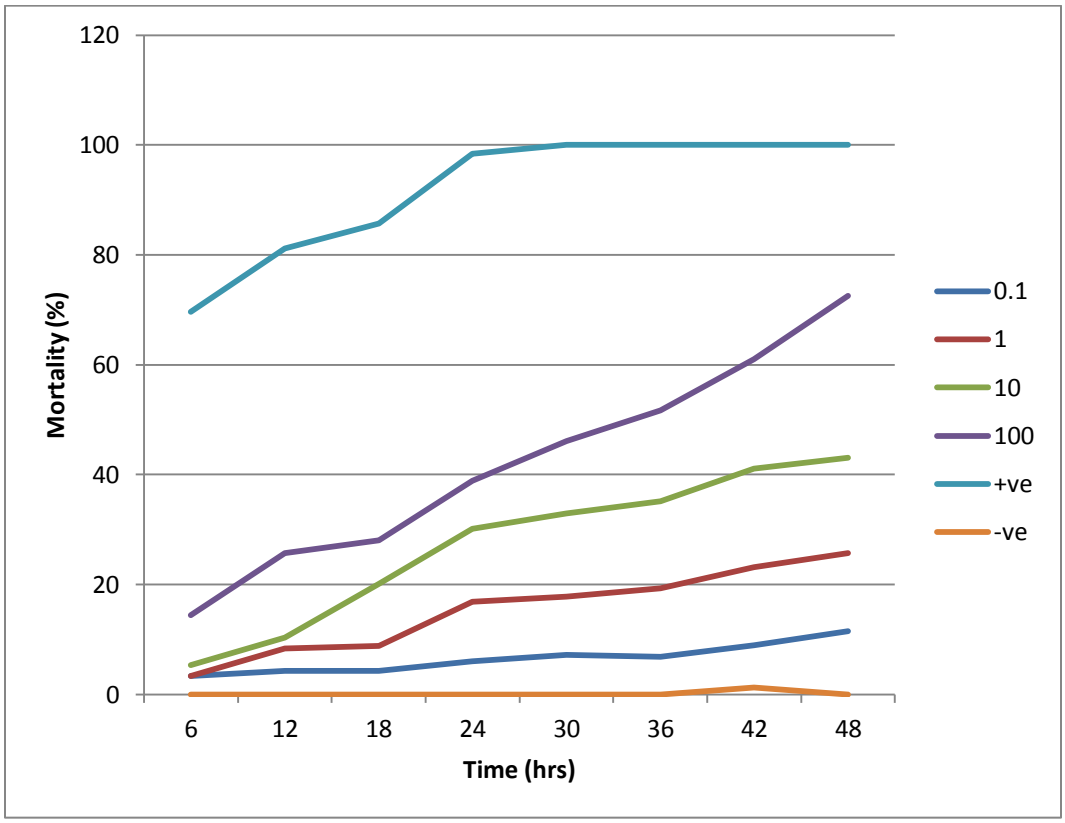

Fig. 3: Mortalities of *Haemonchus contortus* after incubation in leaf methanolic extract of *Azadirachta indica.*

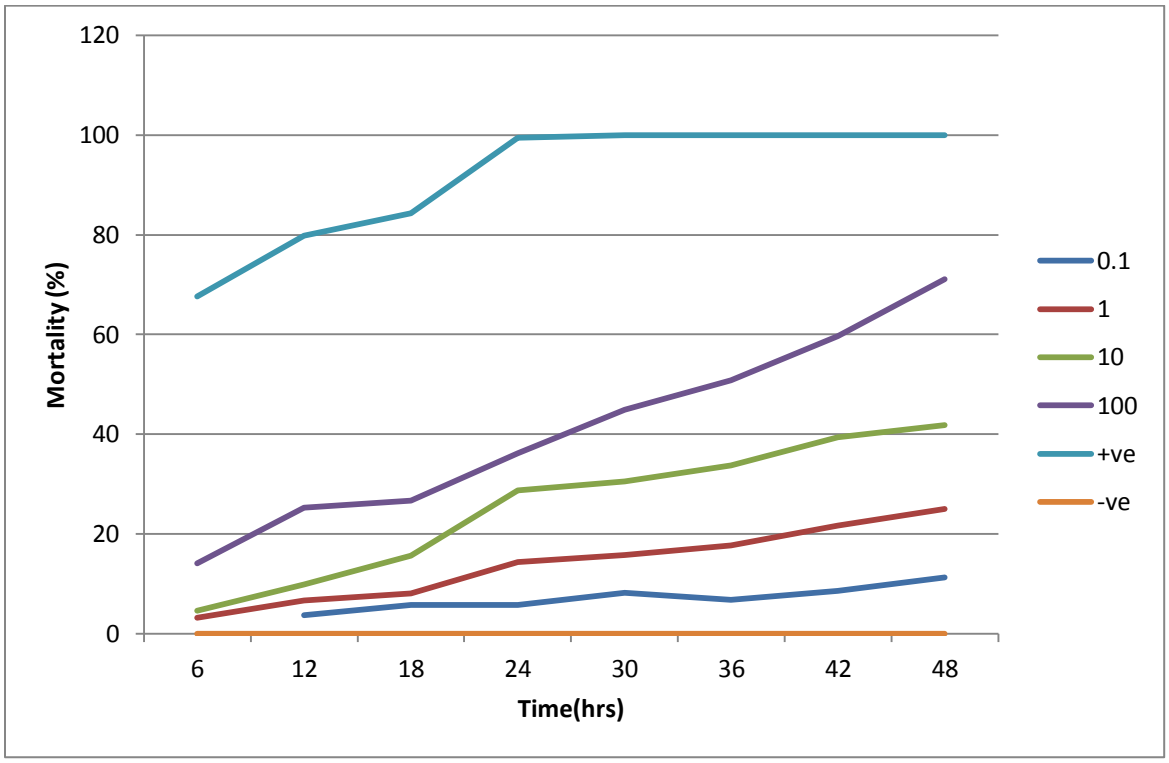

Fig. 4: Mortalities of *Haemonchus contortus* after incubation in stembark methanolic extract of*Azadirachta indica.*

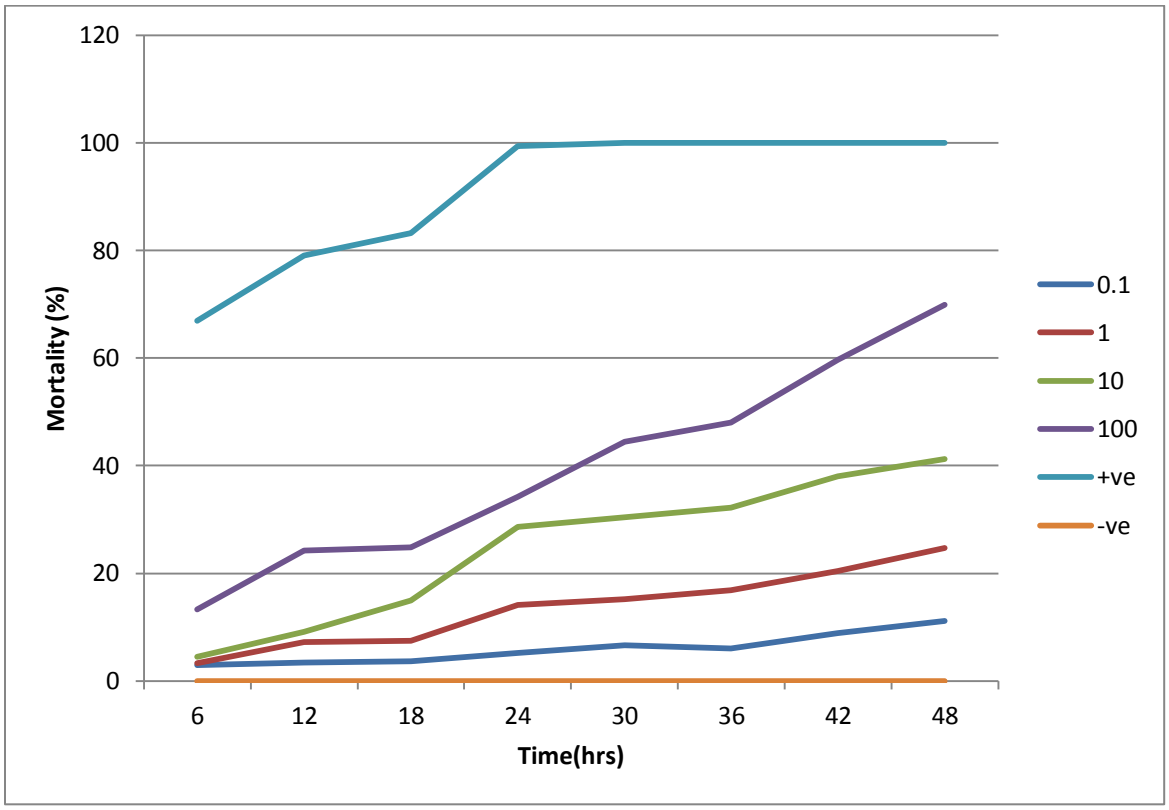

Fig.5: Mortalities of *Haemonchus contortus* after incubation in root methanolic extract of*Azadirachta indica*

Table 3: Summary of the Analysis of Variance (Anova) for the Total Data

	Degree of freedom	Survival (P)	Significance
Plant part	2	0.000000	s
Time	7	0.000000	s
Concentration	5	0.000000	s
Plant part$_{vs}$Time	14	0.921310	ns
Plant part$_{vs}$Concentration	8	0.000293	s
Time$_{vs}$Concentration	28	0.000000	s
Plantpart$_{vs}$Time$_{vs}$ Concentration	56	0.993231	ns

Plate 3: Stained larvae of *Haemonchus contortus*

CHAPTER FIVE

5.1.0 Discussion

The results of the current study revealed the nematocidal potential of methanolicextractsof leaf, stembark and root of *A. indica*(neem) which is in agreement with the results of Katooli *et al.*(2011) and Chitwood(2002) which showed that several families of plants had nematocidal activity consistent with many naturally occurring compounds that are known to possess nematocidal activity.

The phytochemical screeningshowed the presence of carbohydrate, cardiac glycosides, saponins, flavonoids, tannins and alkaloids and absence of anthraquinones derrivatives in stembark, root and leaf of *A. indica*(neem), presence of steroids in leaf and absence in stembark and in root, presence of triterpenes in both stembark and in root and its absence in leaf. Perhaps the *in vitro* effects of the neem plant are due to the presence of the enumerated secondary metabolites

In-vitro studies by Kahiya *et al.* (2003) revealed that condensed tannins from the leaf extract of *Acacia nilotica* inhibited the development of *H. contortus* larvae obtainedfrom goat, also Athanasiadou *et al.* (2001) in *in-vitro* and *in-vivo* studies reported the antihelminthic activity of condensed tannins extracted from Quebracho on the larvae of *H. contortus, Teladorsagia circumcinta* and *Trichostrongylus vitrinus.*

Lahlou (2002) reported that flavonoid is one of the phytochemicals that have antihelminthic effect. In *in-vitro* and *in-vivo* studies, Al-qarawi *et al.* (2001) reported that alkaloids extracted from both the latex and leaves of C*alotropis procera*, was effective in inhibiting the exsheatment of L3 of *H. contortus* to L4 in sheep. Lateef *et al.* (2003) also reported that alkaloids and their glycosides extracted from the root of *Adhatoda vestica*Pwas effective against mixed gastrointestinal infections in sheep. Cardiac glycoside has been shown to be responsible in inducing tonic contraction that resulted in the expulsion of worms from the rats GIT (Kim *et al*., 1992; Hong, 2000).

Steroids were reported to have *in-vitro* antihelminthic activity against adult *Caenorhabditis elegans*, a free-living nematode (Prasharth *et al*., 2001). The presence of steroids in the leaf extract may be responsible for the slightly high nematocidal activity in the leaf when compared to the stembark and root extracts.

The presence of these constituents may havecontributed to the significant nematocidal effect observed.

The quantitative estimation of constituents showed a high percentage in flavonoids followed by alkaloids and tannins. The least quantitative percentage is seen in saponins. The quantity of the constituents may have effect on the activity but the quantity alone may not have accounted for the nematocidal activity observed. Further studies should be carried out in order to isolate each of the constituent.

Phytochemicals in contrast to synthetic pharmaceuticals may exert their effects through the additive or synergistic action of several chemical compounds acting at a single or multiple target sites associated with a physiological process (Kaufmann *et al*., 1999).

The lethal concentration (LC50/ EC) values obtained in the experimental analysis gave a value of 12.30mgml^{-1} for the leaf, 12.58mgml^{-1} for the stembark and 15.84mgml^{-1} for the root extract. The dose concentration at which plant part extracts started having much effect on the parasite was from 10mgml^{-1} to 100mgml^{-1}which is in the range of the LC50 value. The leaf and stem bark performed better than the root extracts, at lower value, 50% of the parasites were killed by the leaf and stem bark extracts when compared to the root extract.

Mortalities recorded were high in the positive control wells with increase in mortality as the time of exposure increases. At 24 hours of parasites exposure to the positive control, there were almost complete mortality of the parasites, none of the parasites survived in the positive control wells from the 30th hour of exposure. In the negative control wells, only one mortality was observed, even in wells with the highesttime of exposure, mortalities were not recorded.

Thisexplains that mortalities recorded were due to the effects of the extracts on the parasites since the parasites thrive well in the negative control wells and could not survive in the positive control wells as well as the extracts. The negative control had no effect on the parasite; mortality observed may be due to stress.

Mortalities of the parasites increased with increase in the concentration and with the time of exposure. At the peak time of exposure that is 48 hours and at the highest concentration of 100mgml^{-1}, mortalities were higher than those of the least time of exposure which is 6 hours and with the lowest concentration of 0.1mgml^{-1}.

Similar to 1000 µg concentration of azadirachtin, mortality of *Haemonchus contortus* larvae was observed by Assis *et al.* (2003) at higher concentrations of plant extracts of hexane and methanol indicating the relevance of this screening test in judging antihelminthic property.*Afzelia africana* and *Combretum molle* extracts demonstrated significant nematocidal activity against *H. contortus* in *in-vitro* egg hatch assay (Simon, 1997).

The variation in larval count could be due to environmental factors and other factors that might have interfered with the results of the studies

The exact mechanism of action as to how the larval activity and worm count is reduced has to be explored. Further studies have to be performed to ascertain the exact mode of action of the inhibitory effect of neem and it's constituents on L3 larvae of *Haemonchus contortus.*

CHAPTER SIX

6.0 Summary,Conclusions and Recommendations

This study shows that methanolic extracts of leaf, stembark and root of *A. indica*(neem) have nematocidal activity indicating the potential for use as herbal remedy. The resultshowed the presence of carbohydrate, cardiac glycosides, saponins, flavonoids, tannins, alkaloids, steroid and triterpenes, but the exact phytochemical responsible for the *in vitro* activity is not known.

The quantitative estimation of the phytochemical constituentsrevealed a high percentage offlavonoids followed by alkaloids and tannins; and the least quantitative percentage in saponins. The lethal concentration (LC50/ EC) analysis gave a least value of 12.30mgml^{-1} for the leaf extract, followed by 12.58mgml^{-1} for the stembark and 15.84mgml^{-1} which is the highest for the root extract.

Mortalities of the parasites increased with increase in concentration and with the time of exposure.

Mortalities recorded were high in the positive control wells with increase in mortality as the time of exposure increases. In the negative control wells, only one mortality was observed, even in wells with the highest time of exposure, mortalities were not recorded.

Although the extracts displayed a good nematocidal activity, further investigation/ research should be carried out in order to understand the real mechanism of action by

which the extracts eliminates the parasites.Further studies should be carried out in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds. This will enable the researcher to know the exact active constituents and its mode of activities on the parasites.

The antimicrobial activities of the plant for the treatments of diseases as claimed by traditional healers should also be investigated.

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Appendix i

Percentage mortality of *Haemonchus contortus* after incubation in leaf methanolic extract of *Azadirachta indica.*

	Time (hrs)							
Conc.mgml^{-1}	6	12	18	24	30	36	42	48
0.1	3.33	4.31	4.31	5.97	7.22	6.81	8.89	11.53
1.0	3.33	8.33	8.75	16.81	17.78	19.31	23.19	25.69
10	5.28	10.28	20.14	30.14	32.92	35.14	41.11	43.06
100	14.44	25.69	28.06	38.89	46.11	51.67	60.97	72.5
+ve	69.58	81.11	85.69	98.47	100	100	100	100
-ve	0	0	0	0	0	0	1.25	0

Appendix ii

Percentage mortality of *Haemonchus contortus* after incubation in stembarkmethanolic extract of *Azadirachta indica.*

	Time (hrs)							
Conc.mgml^{-1}	6	12	18	24	30	36	42	48
0.1		3.75	5.83	5.83	8.1667	6.805	8.611	11.25
1.0	3.194	6.67	8.055	14.44	15.83	17.78	21.67	25.0
10	4.58	9.86	15.69	28.75	30.56	33.75	39.44	41.81
100	14.17	25.28	26.67	36.25	44.86	50.83	59.72	71.11
+ve	67.64	79.86	84.31	99.44	100	100	100	100
-ve	0	0	0	0	0	0	0	0

Appendix iii

Percentage mortality of *Haemonchus contortus* after incubation in root methanolic extract of *Azadirachta indica.*

	Time (hrs)							
Conc.mgml^{-1}	6	12	18	24	30	36	42	48
0.1	2.92	3.47	3.61	5.14	6.67	5.97	8.89	11.11
1.0	3.33	7.22	7.5	14.17	15.14	16.81	20.42	24.72
10	4.44	9.17	15.0	28.61	30.42	32.22	38.06	41.25
100	13.33	24.17	24.86	34.17	44.44	48.06	59.72	69.86
+ve	66.94	79.03	83.19	99.44	100	100	100	100
-ve	0	0	0	0	0	0	0	0