EFFECTS OF KOLAVIRON ON LYMPHOCYTES PROLIFERATION, EXPRESSION OF TOLL LIKE RECEPTOR-2 AND VASCULAR ENDOTHELIAL GROWTH FACTOR-C GENES IN *Wuchereria bancrofti* INFECTED BLOOD

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN BIOTECHNOLOGY

DEPARTMENT OF BIOCHEMISTRY,

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NOVEMBER, 2017

DECLARATION

I hereby declare that the work in this dissertation "**Effects Of Kolaviron On Lymphocytes Proliferation, Expression Of Toll Like Receptor-2 And Vascular Endothelial Growth Factor-C Genes In *Wuchereria bancrofti* Infected Blood**" was performed by me in the Department of Biochemistry, under the supervision of Prof. I.S Ndams and Dr. A. Muhammad. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

Funmilola Elizabeth Audu _____ _____

P13SCBC8045 Signature Date

CERTIFICATION

This dissertation titled "**Effects Of Kolaviron On Lymphocytes Proliferation, Expression Of Toll Like Receptor-2 And Vascular Endothelial Growth Factor-C Genes In *Wuchereria bancrofti* Infected Blood**" meets the regulation governing the award of the degree of Masters in Biotechnology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. I.S. Ndams	_____	_____
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Dr. A. Muhammad	_____	_____
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Prof M.N. Shuaibu	_____	_____
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Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This research work is dedicated to my beloved and indefatigable mother, Mrs Halima Sarki, your love knows no bounds.

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ABSTRACT

The anti-proliferative effect and down regulation of vascular endothelial growth factor C and toll like receptor-2 by kolaviron rich portion of *Garcinia kola* on *Wuchereria bancrofti* infected blood were investigated. Blood samples were collected from consenting volunteers in Talata mafara, Zamfara State, Nigeria, between the hours of 10pm to 12am, stained with Giemsa and viewed microscopically for presence of microfilariae. *Wuchereria bancrofti* positive blood samples were cultured *in vitro* for 72 hours using untreated group as negative control, doxycycline (2µg/ml) treated samples as the positive control while the kolaviron (5µg/ml) treated group was the tested group. Mitotic index, expression of vascular endothelial growth factor-C (VEGF-C), toll like receptor- 2 (TLR-2) were determined using standard procedures, in peripheral blood lymphocytes. Mitotic index was significantly ($P<0.05$) reduced in the kolaviron treated group compared to negative control. Kolaviron also significantly ($P<0.05$) downregulated the expression of VEGF-C and TLR-2 when compared with the untreated group. In both cases, the effects of kolaviron was not significantly different ($P<0.05$) compared to that of doxycycline treated group. Furthermore, strong positive correlations (r) between mitotic index, VEGF-C and TLR-2 expressions were observed. The study concludes that kolaviron rich portion of *Garcinia kola* exhibited anti-proliferative effect and down regulation of VEGF-C and TLR-2 in *Wuchereria bancrofti* infected blood. Thus, the results from this study might have unravelled the potential of kolaviron in the management of complications associated with lymphatic filariasis.

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ABBREVIATIONS

ALB: Albendazole

CDC: Centre for Disease Control

DEC: Diethyl carbamazine

DEPC water: Diethylpyrocarbonate water

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

EDTA: Ethylene diamine tetra acetic acid

GB 1, 2: Garcinia Biflavonoid 1, 2

GPELF: Global Programme to Eliminate Lymphatic Filariasis

IVM: Ivermectin

LF: Lymphatic Filariasis

Mal: myelin and lymphocyte

MDA: Mass Drug Administration

MF: microfilarae

Myd88: myeloid differentiation primary response gene 88

PBMC: Peripheral Blood Mononuclear Cells

PCR: polymerase chain reaction

RPMI: Roswell Park Memorial Institute

TLR 2: Toll like Receptor 2

TNF: Tumour Necrosis Factor

VEGF C: Vascular Endothelial Growth Factor C

WHO: World Health organization

CHAPTER ONE

1.0 INTRODUCTION

Lymphatic filariasis, caused by parasitic *Wuchereria bancrofti*, is a mosquito borne disease characterized by a broad spectrum of clinical manifestation such as temporal/permanent disability and disfiguring leading to severe damage and painful swellings (lymphedema) of the legs and genitals in the late stage of the disease (Hoerauf *et al*., 2011; WHO, 2012; Gomase *et al*., 2013) and eventually stigmatization (WHO, 2013). Although the events leading to the development of chronic pathology in lymphatic filariasis are not fully understood, live filarial parasite and/or their products have a direct effect on lymphatic endothelial cells and in the cells of the innate and adaptive immune system (Nutman, 2013). Vascular endothelial growth factor (VEGF) family which is key regulators of endothelial cell functions has been implicated in lymphangiogenesis and angiogenesis in lymphatic pathology (Pfar *et al*., 2009). Their levels are significantly elevated in individuals with filarial infection both in chronic and microfilaremic states (Bennuru *et al*., 2010). The key mediators when it comes to complications associated with lymphatic filariasis are toll like receptors (TLR). They are pattern recognition factors of the innate immune system responsible for the microbial detection and initiation of the host immune response (Kawai and Akira, 2010). *Wolbachia*, a Gram negative endosymbiont in filarial parasites are key inducers of pro inflammatory cytokines which interact with the immune system through TLR2 thus, contributing to the pathology of lymphatic filariasis (Hise *et al*, 2007).

The World Health Organization (WHO) initiated the Global Programme to Eliminate Lymphatic Filariasis with the goal to eradicate lymphatic filariasis in endemic countries. This initiative utilizes Mass Drug administration, where Diethylcarbamazine, Albendazole or

Ivermectin is administered mass treatment of lymphatic filariasis annually in endemic areas (WHO, 2013). These drugs have been proven not to have good macrofilaricidal efficacy and reported resistance developed by the parasites against the drugs (resistance associated mutation at Tyrosine codon 200 of β tubulin of parasite in albendazole treatment) and they do not alleviate the pathology of the disease (Schwab *et al.*, 2005; Hoerauf *et al*., 2011).

Recently, doxycycline a broad spectrum anti-biotic from the tetracycline family has been used on a single daily dose of 200mg for six weeks in the treatment of Bancroftian filariasis. The treatment resulted in the reduction of plasma vascular endothelial growth factors (Hoerauf, 2008; Bennuru *et al*., 2010), hence the need to use it as our positive control in this study.

Kolaviron is found in defatted ethanol extract of *Garcinia kol*a. The extract is one of the numerous plant products that have been found to have a wide range of medicinal value which include anti-diabetic, anti-inflammatory and anti-proliferative effects amongst other (Farombi and Owoeye, 2011; Ayepola *et a*l., 2014).

In Nigeria, the use of medicinal plants cannot be over emphasized. *Garcinia kol*a is cheaply sourced, readily available and often used for medicinal purposes. Kolaviron is yet to be explored in the management of lymphatic filariasis. Hence, the present study, for the first time, aims to report the role of kolaviron in managing the pathology associated with lymphatic filariasis by focusing on the possible anti-proliferative and the down regulation of toll like receptor 2 and vascular endothelial growth factor C in *W. bancrofti* infected blood lymphocytes. The findings from this work will go a long way in understanding the potential of kolaviron in the management of complications associated with lymphatic filariasis.

1.1 Statement of Research Problem

Nigeria is the third most endemic country in the world with 22.1% prevalence of *W. bancrofti* infection (Nwoke *et al.*, 2010).

Global Programme to Eliminate Lymphatic Filariasis is a mass drug administration intervention, involving the use of Ivermectin, Albendazole and Diethyl carbamazine. This only breaks transmission cycle (WHO, 2013) but do not alleviate the pathology of the disease.

These drugs are reported to have low effects and resistance to these drugs are also emerging (resistance associated mutation at Tyrosine codon 200 of β tubulin of parasite in albendazole treatment) (Schwab *et al.*, 2005; Hoerauf *et al*., 2011)

1.2 Justification for the Study

Lymphatic filariais is of great public health importance as it is one of the major caus of disability (WHO, 2013). The drugs used currently have little effect in ameliorating the pathogenesis of the disease hence the need to look for other means of management. *Garcinia kola* is cheap and locally sourced and understanding its effect on the immunopathology of lymphatic filariasis could be a stepping stone in the development of therapeutic drug in the management of Lymphatic filariasis or possibly in the production of target based DNA vaccine. There is no report yet on the effect of kolaviron on Lymphatic filariasis. The effect of Kolaviron on lymphatic filariasis is yet to be studied; since reports have shown its anti-inflammatory and anti-proliferative effect, kolaviron may be potent in the management of lymphatic filaraisis.

1.3 General Aim

To investigate the effects of Kolaviron on the immunpathology of Lymphatic Filariasis.

1.4 Specific Objectives

i. To determine the anti–proliferative effect of kolaviron on the cultured peripheral blood lymphocytes of *Wuchereria bancrofti* infected blood using Mitotic index assay.

ii. To determine the effects of Kolaviron on the expression of toll like receptor-2 genes in the cultured peripheral blood lymphocytes sample using Real time-PCR.

iii. To determine the effects of Kolaviron on the expression of vascular epithelial growth factor- C (VEGF C) gene in the cultured peripheral blood lymphocytes sample using Real time-PCR.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lymphatic Filariasis

Lymphatic filariasis, a mosquito borne disease, is a disabling parasitic disease that has been identified by the World Health Organization (WHO) as a major public health issue with increasing prevalence worldwide (WHO, 1992). The infection is usually acquired in childhood while its indication, that is, pathophysiology occurs later in life causing temporary/permanent disability which may include severe damage and painful swelling, disfiguring swelling of the leg and genitals developing in the late stage of the disease (Gomase *et al*., 2013).

Lymphatic filariasis is caused by thread-like parasitic worms called filariae. They belong to the super family *Filaroidea* and family *Onchocercidae*. *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* are responsible for the global burden of lymphatic filariasis. The specie *Wuchereria bancrofti* is the most prevalent worldwide accounting for about 90 percent of cases, *Brugia malayi* is found mostly in eastern Asia and *Brugia timori* is confined to East Timori and adjacent islands accounting for about 10 percent of cases (Micheal *et al*., 1996, Al meida *et al*., 1999; Hoerauf *et al*., 2011).

The worms have an estimated active reproductive span of 4–6 years, producing millions of small immature larvae (microfilariae) which circulate in the peripheral blood. They are transmitted from person to person by several species of mosquito such as *Aedes*, *Anopheles*, *Culex* and *Mansonia* (WHO, 2013).

2.2 Wuchereria bancrofti

Wuchereria bancrofti is a nematode causing lymphatic filariasis throughout the tropics and sub-tropics. There are two strains of *W. bancrofti*; the nocturnal periodic strain which is widely distributed in endemic regions with the microfilariae being in their highest concentrations between the hours of 10pm and 2am and the sub-periodic strain which is found in the Pacific region, and has microfilaraemia all the time with the highest numbers being detected between noon and 8pm (WHO, 1992).

Humans are the only known reservoir host of *W. bancrofti*. In humans, the microfilariae show a characteristic periodicity in the course of the 24 hour cycle. They live mainly in the pulmonary capillaries, from where a proportion of them escape into the peripheral blood and can be detected during the hours of their periodicity (CDC, 1993; Bockarie *et al*., 2009).

The appearance of microfilariae in the peripheral blood of human synchronizes with the biting period of the vector mosquito and depends upon the sleeping habits of man. When filarial adult worms are lodged in lymphatic and in lymph glands, they obstruct mechanically the flow of lymph and also produce inflammatory and allergic reactions (Sarojini and Senthikumar, 2013).

The tail of the microfilariae of *W. bancrofti* tapers to a delicate point and exhibits no terminal nuclei and can thus be easily distinguished from the microfilariae of *Brugia malayi* and *Loa loa*, the other sheathed microfilariae of clinical importance. The sheath of the microfilariae of *W. bancrofti* stains pink with Giemsa. The microfilariae are 260 μm × 8 μm in length (Simonsen, 2008).

The adult worm inhabits the lymphatics and the female produces sheathed microfilariae which circulate in the peripheral blood. The mosquito acquires the infection by ingestion of the

microfilaria in the blood meal (O'Connor *et al.*, 2003). The ingested microfilariae lose their sheath on arrival in the stomach of the mosquito which then migrate to the thoracic muscles and develop into infective larvae over a period of 10 - 14 days. The larvae then migrate to the mouth parts of the mosquito and enter the skin of the definitive host through the puncture wound when a blood meal is taken. The infective larvae enter the peripheral lymphatics where they grow to mature male and female worms. After mating, the gravid females release sheathed microfilariae which can be detected in the peripheral blood 8 - 12 months after initiation of infection (Simonsen, 2008). The periodicity of the parasite aided in the sampling for the experiment as positive samples were only detected at night.

Figure 2.1 Microfilaria of *Wuchereria bancrofti* in thick blood film.

Source: https://www.cdc.gov‹ dpdx ›lymphaticfilariasis

2.2.1 Life Cycle of *Wuchereria bancrofti*

Filarial parasites develop in two hosts (biphasic cycle) involving definitive mammalian host and various genera of mosquitoes (*Anopheles*, *Aedes*, *Culex* and *Mansonia*). *Wuchereria bancrofti* appears to be an exclusive human parasite. Man is the primary host (definitive) whereas mosquito acts as the Secondary hosts (intermediate) (Bockarie *et al*., 2009 and Sarojini *et al*., 2013). Development of the parasite in human takes a long time (8-12 months) but in mosquitoes it takes 10-14 days only (Sarojini and Senthikumar, 2013).

The adult worms (thread like, 4-10 cm long) are lodged in the lymphatic system of man. The female and male worms mate within the human body and the fertilized female liberates thousands of larvae, known as microfilariae (mf). During day time microfilariae remain concentrated in the capillaries and blood vessels of internal organs especially lungs which are then released into the blood stream and circulated in the peripheral blood at night periodically. Further development of microfilariae takes place in the body of the mosquito vector (Weil *et al*., 1997; Sarojini and Senthikumar, 2013).

The microfilaria (mf) ingested by mosquitoes along with the blood, sheds its body cover and migrates to the thoracic muscle of the mosquito, where it undergoes two moultings L1 stages or 1st stage; which is short, thick and sausage form; L2 stage or 2nd stage which is long with slow movement and L3 stage or 3rd stage, the infective larva stage. After the second moulting where the parasite loses its cuticle (L3 stage), the larva migrates to the proboscis (mouth parts) of the mosquito. When the infected mosquitoes feed on man, these L3 larva are deposited on the skin near the site of the bite, few of them succeed in penetrating the wounds. The infective stage

larvae develop into adult worm within human body (Micheal and Bundy, 1997, Simenson, 2008; Sarojini and Senthikumar, 2013).

The larvae mature over a period of months into lymphatic-dwelling adult worms which mate and release microfilariae into the host's blood stream, ready to be passed by a blood meal to the mosquito. In the mosquito, infective larvae range from 0.8 to 1.5 mm. Due to their significant size; it carries only a limited number of larval parasites while feeding on a prospective host (Michael and Bundy, 1997). Therefore, exposure to infective larvae must be intense and prolonged for infection to occur.

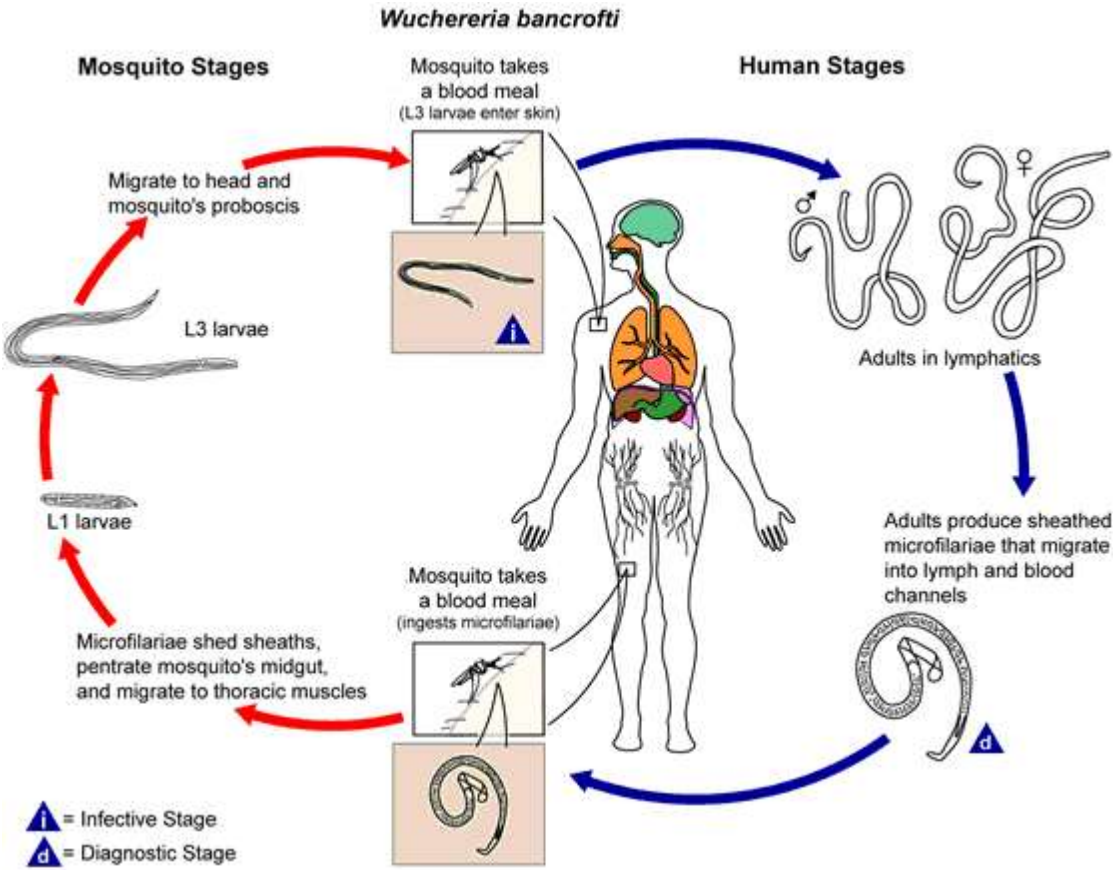


Figure 2.2 Lifecycle of *Wuchereria bancrofti* (WHO, 1992)

2.3 Epidemiology of Lymphatic Filariasis

It is estimated that close to 120 million people are infected with filarial parasites in 73 countries in the tropics and sub-tropics, while 1.39 billion people live in areas where filariasis is endemic (WHO, 2007; GPELF, 2011). One third of the people affected with the disease live in India, one third in Africa and most of the remainder in South Asia, the Pacific and the Americas (GPELF, 2011).

Lymphatic filariasis causes profound physical and social disability and it is estimated that over 40 million people are living with lymphatic disability mainly due to swelling of the limbs, male genitals, while to a lesser extent, of the breasts and female genitals (CDC, 1993). A further 76 million people have hidden internal damage to their lymphatic and renal systems (Bockarie *et al*., 2009).

Lymphatic filariasis infection can occur early in life. In some areas, about 30% of children are infected before the age of 4 years; while the clinical disease usually appears later in life, subclinical damage starts at an early age (Simonsen, 1996). Lymphatic filariasis is unlikely to cause lymphoedema or hydrocoele in children less than 10–15 years of age (Witt and Ottesen, 2001).

In Africa, about 38 countries are thought to be affected by Lymphatic filariasis with the parasite being exclusively *W. bancrofti* (Mbah and Njoku, 2000). Nigeria is the third most endemic country in the world with 22.1% of the population thought to be infected (Michael and Bundy, 1997). In Nigeria, Lymphatic *filariasis* has been reported in rural communities in the lower Cross River Basin (Okon *et al*., 2010), Ezza in Ebonyi State (Anosike *et al*., 2005), Igwun basin of

Rivers State with prevalence of 15.5% and parts of the Niger Delta with hydrocoele prevalence of 12.3% (Agi and Ebenezer, 2009), 20% of studied population in Oguta Local Government Area of Imo State had Lymphatic filariasis (Ojiako and Onyeze, 2007), parts of Central Nigeria including rural communities in Plateau and Nassarawa States with an overall prevalence of 12.3% (Abel *et al*., 2002, Eigege *et al*., 2003). Lymphatic filariasis is also endemic in three villages in Kano state with the prevalence of over 1% (Dogara *et al*., 2012).

2.4 Pathophysiology and Clinical Features of Lymphatic Filariasis

Lymphatic filariasis is a disease characterized by a broad spectrum of clinical manifestations seen among the majority of the infected people (Nutman and Kumaraswami, 2001).

The lymph nodes and the lymphatic vessels are the primary sites where the developing and adult worms of *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* locate. These are the sites where pathology and the pathological processes associated with the infection are most intense (Mak, 2012).

Disease condition in lymphatic filariasis is both acute and chronic. The acute manifestations are typically characterized by retrograde adenolymphangitis which is accompanied by systemic signs of inflammation including fever and chills and are thought to result primarily from bacterial and fungal infections caused by compromised lymphatic function and inflammation of lymph nodes and lymphatic (i.e. lymphadenitis and lymphagitis) (Dreyer *et al*., 1999).

In Bancroftian filariasis, the main clinical features are hydrocele, lymphedema, elephantiasis and chyluria whereas for Brugian filariasis the urogenital areas are commonly spared. The development of pathology is thought to be dependent on the presence of the adult worm.

Histologically, the worm elicits little reaction as long as it is alive; however, upon death of the worm, adverse reaction follows (Dreyer *et al*., 2000; Figueredo-Silva *et al*., 2002).

In pathogenesis of lymphatic filarial disease (lymphedema, hydrocele, and elephantiasis), live filarial parasites and/or their products have a direct effect on lymphatic endothelial cells and on the cells of the innate and adaptive immune system. Thus, interplay among inflammatory/ immune mediators, wearing away of the parasites, *Wolbachia* and other factors contribute to pathogenesis and development of filarial disease with secondary microbial infections further aggravating the pathology (Nutman, 2013).

The two major independent components of lymphatic filarial disease are lymphangiectasia and inflammatory reactions around the adult worms which is not restricted to the exact segment of lymphatics where the worms reside (Ong and Doyle, 1998). This suggests that the inflammatory process is mediated by soluble products excreted and/or secreted by the parasite which act on the lymphatic endothelial cells, thus, leading to a stage of irreversible lymphatic dysfunction (Olszewski *et al.*, 1997; Fugueredo-Silva *et al.*, 2002). These chronic phases of lymphatic filariasis develop years after initial infection (Pani *et al*., 1995).

In men, scrotal hydrocele is the most common chronic clinical manifestation of bancroftian filariasis (Dreyer *et al*., 2000). In some endemic communities, 40–60% of all adult males have hydroceles. Hydroceles are due to accumulation of edematous fluid in the cavity of the tunica vaginalis testis. Though the mechanism of fluid accumulation is unknown, evidence indicates that in bancroftian filariasis, the scrotal lymphatics are the preferred site of localization of the filarial worms and their presence may stimulate not only the proliferation of lymphatic

endothelium but also a transudation of hydrocele fluid whose chemical composition is not dissimilar to serum (Nutman, 2013).

Tropical pulmonary eosinophilia (TPE) is a distinct syndrome that develops in some individuals infected with *W. bancrofti* and *Brugia malayi* characterized by nocturnal paroxysmal cough and wheezing (Ong and Doyle 1998). Other symptoms associated with lymphatic filarial disease are variety of renal abnormalities including hematuria, proteinuria, nephrotic syndrome and glomerulonephritis; Circulating immune complexes containing filarial antigens have been implicated in the renal damage. Lymphatic filariasis may also present as a mono-arthritis of the knee or ankle joint (Adebajo, 1996; Melrose, 2002).

Reductions in haematological parameters (total red blood cells, lymphocyte, basophils, monocytes, mean corpuscular haemoglobin) were observed in filariasis affected patients when compared with normal patients. Total white blood cell count, neutrophils, eosinophils, platelet count and mean corpuscular value was increased in affected patients (Sarojini and Senthikumar, 2013). Reductions in haematological parameters may be due to immune system modulation by the filarial antigen or the response of the innate immune system to curb the infection. Lymph vessels are usually red hot, painful and tender in infected patients.

2.5 Immunopathology of Lymphatic Filariasis

The presence of *Wolbachia* endosymbionts, Gram negative bacteria, in all stages of lymphatic filarial parasites has provided new insight on the adverse reactions associated with anti-filarial chemotherapy. Inflammatory molecules mainly lipopolysaccharide (LPS)-like molecules released from endosymbionts on death of the parasites are largely responsible for the adverse reactions encountered during anti-filarial chemotherapy. Pathological responses depend not only on exposure to filarial antigens/infection, but also on host-parasite endosymbiont factors and to intervention with antifilarial treatment. Treatment induced or host mediated death of parasites are associated with various grades of inflammatory response, in which eosinophils and LPS from endosymbionts play prominent roles, leading to death of the parasite, granulomatous formation and fibrosis (Mak,2012, and Nutman, 2013).

The canonical host immune response to filarial parasite involves the T-helper 2 (Th2) and involves the production of cytokines (Allen and Maizels, 2011). Host innate immune response (macrophages, eosinophil, and neutrophils) also attack filarial parasites in the lymphatic and lymph nodes (Lawrence and Devaney, 2001). Filarial pathology is characterized by high levels of circulating immune complexes and pro inflammatory cytokines (Senbagavalli *et al*., 2011). Pro inflammatory cytokines (Interleukin 6 and Tumour necrosis factor α) of innate origin plays a role in the initiation of pathology in filarial infected animal model. Patients with both acute and

chronic manifestations of lymphatic filariasis have elevated levels of interleukin 6 while those with the chronic disease have elevated levels of tumor necrosis factor (Cuenco *et al*., 2004).

In lymphatic filariasis, the pathogenesis of lymphoedema and hydroceole might be influenced by host genetic factor. Studies in Hiati suggested a genetic basis for developing pathology in lymphatic filariasis; showing that 42% of patients with lymphedema in at least one leg had parents with lymphedema (Cuenco *et al*., 2004). Another study implicated Toll like receptor 2 polymorphisms in susceptibility to infection (Junpee *et al*., 2010). A case control study of the role of vascular endothelilal growth factor A (VEGF-A) single nucleotide polymorphisms in hydrocele showed that VEGF-A gene polymorphism in -460C/T was significantly associated with higher levels of plasma VEGF-A and development of hydrocele (Debra *et al*., 2007).

2.6 Vascular Endothelial Growth Factor C (VEGF-C)

Vascular endothelial growth factors (VEGFs) are key regulator of endothelial cell functions required for vasculogenesis and also for physiologic and pathologic angiogenesis (Carmeliet, 2000). Recent studies have implicated the VEGF family in lymhagiogenesis (Pfar *et al*., 2009). Recently, it was shown that vascular endothelial growth factor-C (VEGF C) levels are significantly elevated in individuals with filarial disease both in the individual with the overt pathology and microfilaremic individuals (Bennuru *et al*., 2010).

Genetic model in rats using transgenic keratin-14 promoter (these rats lack functional cutaneous lymphatic system hence lymphedema formation in the skin) showed the expression of VEGF-C being up-regulated by the pro- inflammatory cytokine (interleukin and tumor necrosis factor α) suggesting that pro-inflammatory cytokine could affect the lymphatic vessels via VEGF-C (Ristimaki *et al*., 1998; Makinen *et al*., 2001).

VEGF-C controls lymphagiogenesis by activating the VEGF receptor-3(VEGF-R3) which is primarily expressed in the lymphatic endothelium (Achen *et al*., 1998). The binding of VEGF-C and its receptor is important for lymphatic proliferation and it is believed to be involved in dilation of the lymphatics and development of lymphedema in patients with lymphatic filariasis (Debrah *et al*., 2006). Observations also suggest that VEGF-C/VEGF-R3 interactions are the principal mechanism for lymphagiectasia in filarial infection (Jeltsch *et al*., 1997 and Debrah *et al*., 2006).

Wolbachia an endosymbiont of *Wuchereria bancrofti* and *Brugia malayi* may be a major factor in the initiation of pro inflammatory response and the increased production of VEGF-A and VEGF-C (Pfar *et al*., 2009). Studies have shown that wolbachia predominantly activate the receptors Toll like receptor 2 and Toll like receptor 6 which resulted in signaling through adapter proteins MyD88 and Mal, although the mechanism of activation is yet to be clarified (Hise *et al*., 2007).

Recently, it was demonstrated that the increased levels of VEGF-C observed in patients were reduced following treatment with doxycycline (Debrah *et al*., 2007). Wolbachia was also depleted suggesting that it may play a major role in the release of VEGFs because the endobacteria are known to induce proinflammatory cytokine (Brattig *et al*., 2000).

2.7 Toll -Like Receptor 2

Toll-like receptors (TLR) are pattern-recognition receptors of the innate immune system that form a key component of microbial detection and are important in the initiation of host immune responses (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2010). TLR are involved in the recognition of a wide spectrum of pathogens by binding to pathogen-associated molecular

patterns. Upon binding to their cognate ligands, TLRs activate the innate immune response, leading to the production of proinflammatory cytokines and chemokines (Iwasaki and Medzhitov, 2004).

Toll-like receptors dependent pro-inflammatory cytokine, if left unregulated, can spread to cause severe filarial pathology (Lohela *et al*., 2009). Studies of animal models of filarial infection and *in vitro* studies in humans have suggested that *Wolbachia*-derived molecules from filarial parasites are key inducers of pro-inflammatory cytokines which interact with the immune system through TLR2 and contribute to the pathology of lymphatic filariasis (Brattig *et al*, 2004; Hise *et al*, 2007).

Toll like receptor 2 may lead to increased production of angiogenic factors through direct effect on vascular endothelial growth factor and angiopoietin production and/or indirect effect due to TLR induced pro inflammatory cytokine production (that is, Tumor necrosis factor α and interleukin 17) (Babu *et al*., 2012). Thus, toll like receptor 2 are key factors in lymphatic filarial pathology.

2.8 Management and Control of Lymphatic Filariasis

The main intervention measure recommended for the control of Lymphatic Filariasis is mass drug treatment of the human population (with combination of Ivermectin and Albendazole), with vector control serving a supporting role when feasible and affordable (Ottesen *et al.,* 1997). Mass drug administration (MDA) programs have been implemented that annually treat the majority of the eligible endemic populations with drugs that reduce peripheral (blood or skin) microfilarae loads for six or more months (WHO, 2007). Three of such drugs have been introduced to combat filariasis: Diethylcarbamazine, Ivermectin and Albendazole.

2.8.1 Diethyl Carbamazine

Diethlycarbamazine, the piperazine derivative, attacks filarial parasites at all stages of the parasite life-cycle. The mode of action of diethylcarbamazine is still not completely understood, but it results in the sequestration of microfilariae and their eventual destruction by the immune system, and is dependent on inducible nitric oxide synthase and cyclooxygenase (Mcgary *et al*., 2005).

2.8.2 Ivermectin

Ivermectin is an analogue of avermectin, which belongs to a family of 16-membered macrocyclic lactones. It is known to increase membrane permeability to chloride ions possibly as a result of their interaction with chloride ion channels. Its broad spectrum of activity and wide safety margin has made it the drug of choice for nematode and arthropod parasitism in cattle, sheep, goat, swine and horses (Ademola *et al*., 2003). Ivermectin acts by hyper polarization of glutamate-sensitive channels which results in paralysis of microfilaria. It was recently shown to block the contractile activity of the excretory/secretory vesicle. As a result, molecules that may modulate the immune response are not released, leaving the microfilariae undefended in the lymph nodes (Omura and Crump, 2004).

2.8.3. Albendazole

Albendazole is a carbamate benzimidazole, broad spectrum anthelminthic drug which inhibits the polymerization of worm β-tubulin and microtubule formation. A single dose of 400 mg decreases *Wuchereria bancrofti* microfilaraemia for 6–12 months, and when it is used in combination with Ivermectin (or Diethylcarbamazine), the numbers of MF in lymphatic filariasis are reduced for much longer times than after a single dose of Albendazole. Although higher doses may have effects on adult *W. bancrofti*, it is not clear whether the low 400-mg dose would

work synergistically with diethylcarbamazine against adult worms (Critcheley *et al*., 2005; Taylor *et al*., 2010).

2.8.4 Doxycycline

Doxycycline is a broad spectrum anti-biotic from the tetracycline family. All studies in Bancroftian filariasis used a single daily dose of 200mg of doxycycline (Hoerauf, 2008). Six (6) and four (4) weeks regimen showed a comparable marofilaricidal activity of 92% and 83% respectively (Debrah and Marfo, 2006; Debrah *et al*., 2007). Doxycycline interrupts embryogenesis and also ameliorates the effect of the disease; the diameter of scrotal lymph vessels, a marker for lymphangiogenesis induced by filarial worms is reduced after treatment with doxycycline (Taylor *et al*., 2005; Debrah *et al*., 2006).

Reduction in VEGFs which is essential for angiogenesis and lymphangiogenesis (VEGF A and C respectively) were noticed (Adams and Alitalo, 2007). Doxycycline has also been shown to halt/reduce lymphoedema stages (Dreyer *et al*., 2002). This effect may have been due to reduction in pro inflammatory cytokines and/or prevention of filarial antigen from modulating immune response.

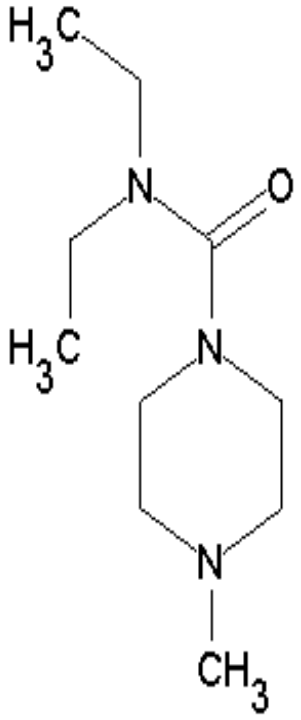


Figure 2.3: Chemical structure of diethylcarbamazine

Source: https://pubchem.ncbi.nlm.nih.gov›diethycarbamazine

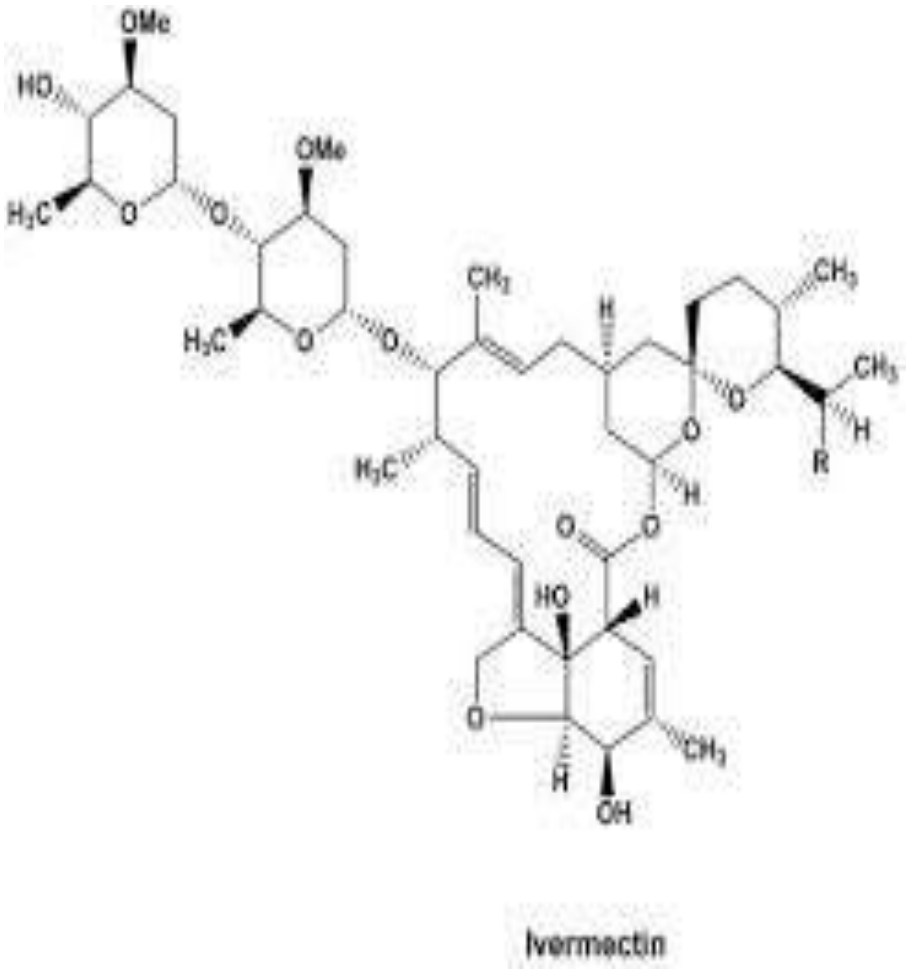


Figure 2.4: Chemical structure of Ivermectin

Source: https://pubchem.ncbi.nlm.nih.gov›ivermectin

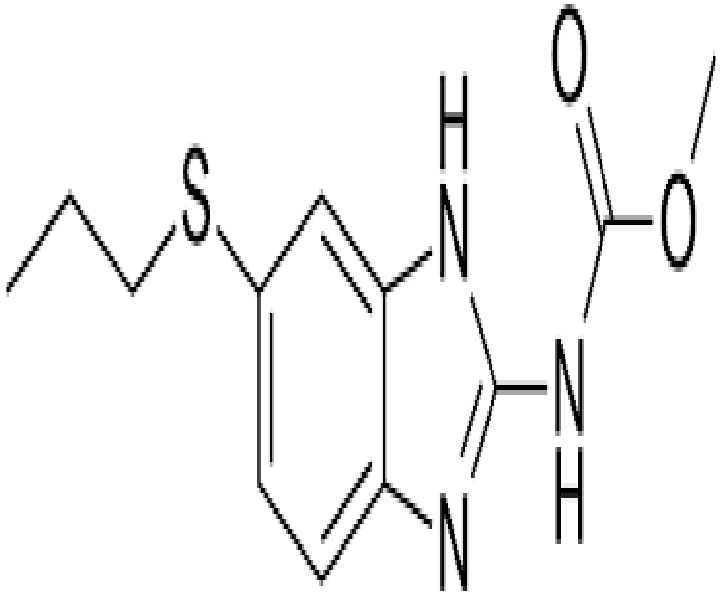


Figure 2.5: Chemical structure of Albendazole

Source: https://pubchem.ncbi.nlm.nih.gov›albendazole

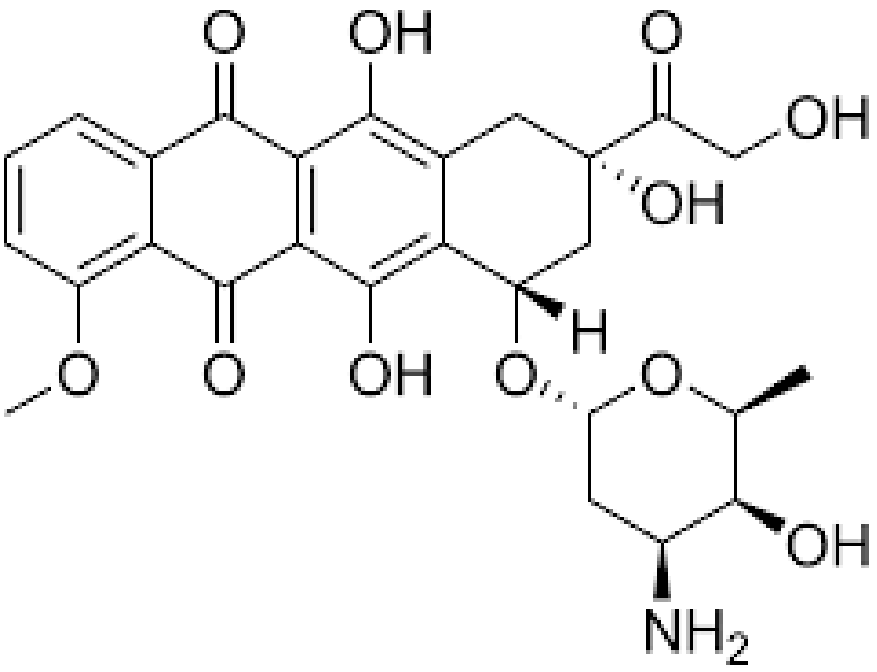


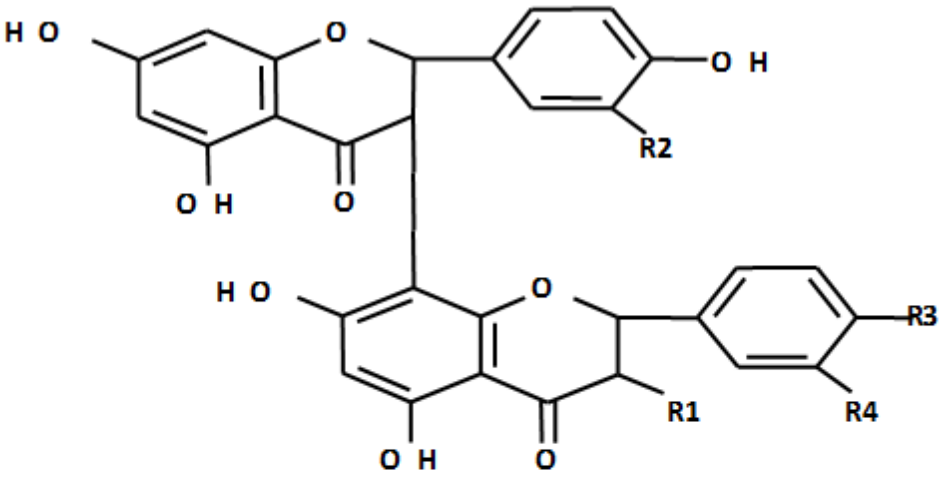
Figure 2.6: Chemical structure of Doxycycline

Source: https://pubchem.ncbi.nlm.nih.gov›doxycycline

2.9 Kolaviron

Kolaviron is a compound found in defatted ethanol extract isolated from the seeds of *Garcinia kola* commonly called Bitter kola ('Orogbo' in Yoruba, 'Namijin goro' in Hausa and 'Aku ilu'). This seed is highly valued in West and Central Africa for its edible nuts with the belief that it promotes longevity. Extracts of the plant are used in traditional African medicine for the treatment of laryngitis, cough and liver diseases. It is a mixture of three compounds - Garcinia biflavonoid GB1, Garcinia biflavonoid GB2 and Kola flavanone in ratio 2:2:1 (Iwu *et al*, 1990).

The activity of kolaviron may be related to the presence of the biflavonoid group. The biflavanones of *Garcinia kola* are pharmacologically active with several pharmacokinetic advantages over simple monomeric flavonoids (Farombi *et al*., 2000). The Anti-inflammatory and hepatoprotective property of kolaviron has been extensively studied. Anti-inflammatory activity of kolaviron was reported in rats treated with 100mg/kg kolaviron with edema inhibition of 59%; it is also reported to have an anti-biotic property (Farombi *et al*., 2000; Hong-xi and Song, 2001). Kolaviron prevent hepatotoxicity and has anti-oxidant activity both in vivo and in vitro (Adaramoye *et al*., 2005, Adaramoye and Adeyemi, 2006). It has also been reported that kolaviron normalized serum levels of inflammatory markers in diabetic rats although no significant difference was shown in VEGFs and IL1β in normal rats compared the untreated control and also has anti genotoxic effects (Farombi *et al*., 2005 and Omolola *et al*., 2014).



	R1	R2	R3	R4
GB1	**OH**	**H**	**OH**	**H**
GB2	**OH**	**H**	**OH**	**OH**
Kolaflavanone	**OH**	**H**	**OMe**	**OH**

Figure2.7 Chemical Structure of Kolaviron (Farombi e*t al*., 2000)

Figure 2.8 *Garcinia kola* seeds

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

RNA extraction kit, Reverse transcription kit and real time PCR kit (Bioneer Corporation, Daejeon, Korea); Ficoll−paque (GE health care life sciences, USA), Sterile Phosphate buffer saline, Penicillin-streptomycin solution, Fetal Bovine serum (Sigma Aldrich, USA), Complete RPMI media (with L- Glutamine) (Sigma Aldrich, USA), 5% Giemsa, colcemid, potassium chloride (KCl), Phytohaemaglutinin (Sigma Aldrich, USA). Doxycycline (Doxycap, Hovid pharmaceuticals, Malaysia) was purchased from a local Pharmacy (Kaduna, Nigeria). *Garcinia kola* was purchased from a local market (Kaduna, Nigeria) and authenticated at the Herbarium unit of the Department of Botany, Faculty of Science, Ahmadu Bello University, Zaria with voucher number V/N5105.

3.1.2 Equipment

Centrifuge machine, EDTA vacutainers, sterile centrifuge tubes, auto pipettes, culture plates, haematocytometer, vortexer, tissue culture hood (Baker Sterilgard, model SG400M), CO_2 incubator (Forma scientific, model 3193), microscope (Olympus, Japan), RT- PCR machine (lightcycler, Roche, version 3.5, Basel, Switzerland).

3.1.3 Ethical clearance

Ethical clearance was sought and collected from Zamfara State Ministry of Health with the informed consent of the volunteers.

3.1.4 Experimental set up/ Treatment

Six (6) volunteers screened and found infected with *W. bancrofti* were used for this research and grouped as follows; Control group: infected and untreated culture

Kolaviron (Test) group: culture treated with 5µg/ml Kolaviron

Doxycycline (positive control) group: culture treated with 2µg/ml

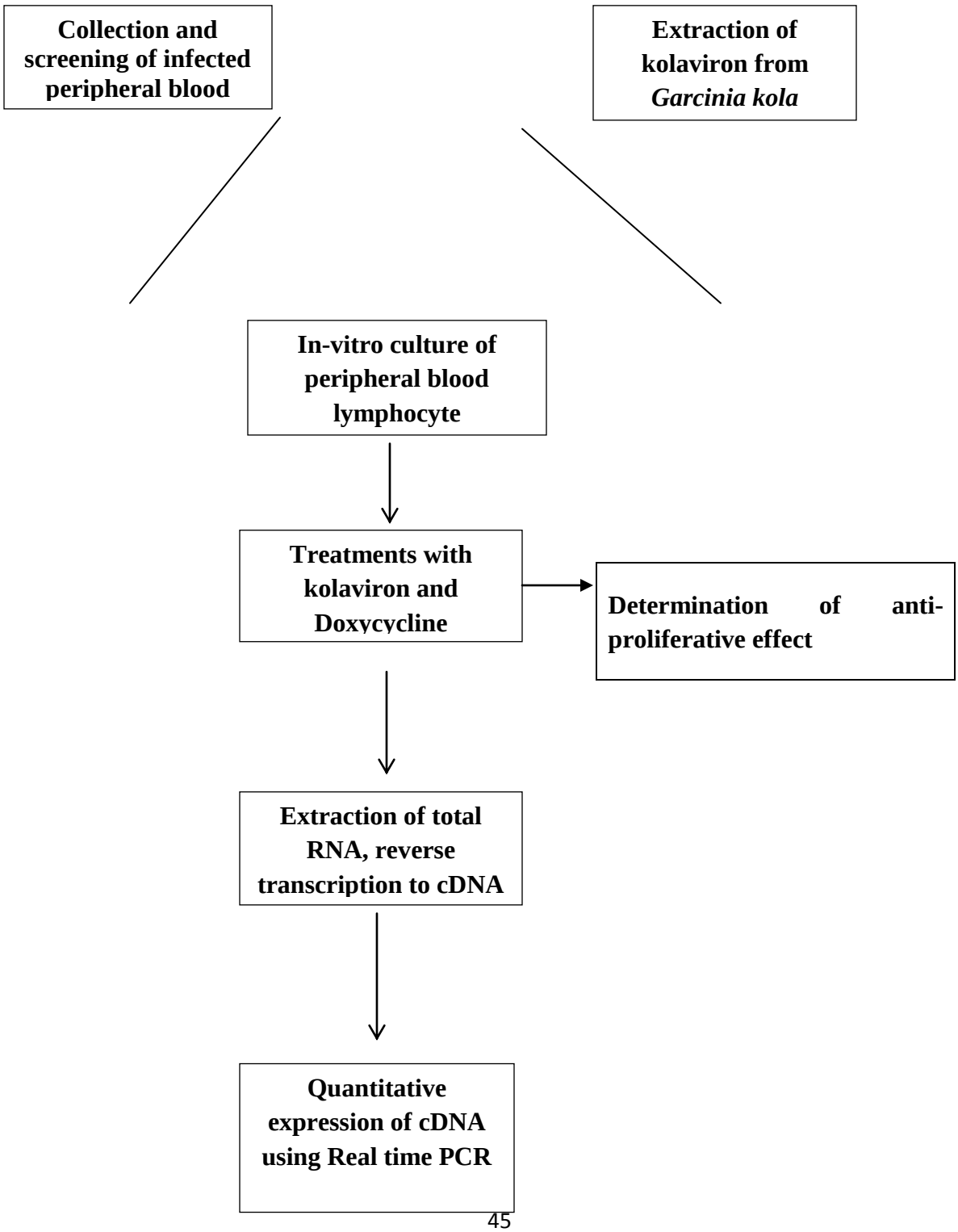

Collection and screening of infected peripheral blood
Extraction of kolaviron from *Garcinia kola*
In-vitro culture of peripheral blood lymphocyte
Treatments with kolaviron and Doxycycline
Determination of anti-proliferative effect
Extraction of total RNA, reverse transcription to cDNA
Quantitative expression of cDNA using Real time PCR

Figure3.1. Experimental design

3.2 Methods

3.2.1 Identification of parasite

Peripheral blood was collected from volunteers intravenously within the hours of 10pm to 12am as parasites are nocturnal (Simonsen, 2008). Intravenous blood was collected from volunteers into heparin vacutainer and within three hours of sample collection, three drops (150µL) of blood was taken consistently using a dropping pipette on a glass slide and an oval shaped thick smear was made with the help of corner of other slide, it was allowed to dry and stained with Giemsa stain thereafter. Slide was observed under the oil immersion lens by putting one drop of oil (X 100). *Wuchereria bancrofti* microfilariae were detected based on their morphological feature that is, has rounded anterior end and tapering tail with both ends free of nuclei, the sheath also stains pink with Giemsa stain (Simonsen, 2008).

3. 2. 2 Extraction of Kolaviron

Kolavion was extracted using the method as described by Olaleye *et al*., 2000. Kolaviron was extracted from the fresh seeds of *Garcinia kola*; (50g) powdered seeds were extracted with 250ml of light petroleum ether (boiling point 40–60° C) in a soxhlet extractor for 24 hr. The defatted, dried marc was re-packed and then extracted with 250ml of acetone. The extract was concentrated and diluted to twice its volume with distilled water and was extracted with ethyl acetate (6:250). The concentrated ethyl acetate fraction gave a yellow solid known as kolaviron. Kolaviron was separated by thin layer chromatography (TLC) using silica gel GF 254 coated plates and solvent mixture chloroform/methanol (80:20). TLC revealed the presence of three compounds GB1, GB2 and kolaflavanone in a ratio 2:2:1, and were identified by their R_f values compared with reference compounds.

3.2.3 Isolation of Peripheral blood mononuclear cells (PBMC)

This was carried out as described by the manufacturer's protocol (GE healthcare life sciences). To a 10ml centrifuge tube, 2ml of anticoagulant treated blood from volunteers was added and an equal volume of phosphate buffer saline was added to make up the total volume of 4ml. The blood and buffer was mixed together by inverting the tube several times. 3ml of Ficoll plaque was added to the centrifuge tube and carefully layered on the diluted blood sample. It was then centrifuged at 400 ×g for 30 minutes without brakes at 20^{o}C. The peripheral blood mononuclear cells (PBMC) layer was then transferred using a sterile pipette.

3.2.4 Peripheral blood mononuclear cell culture

Peripheral blood mononuclear cells (PBMC) were cultured using Roswell Park Memorial Institute medium 1640 (RPMI 1640 medium). 0.5ml (500µl) of the sample was added to 4.5 ml of culture medium. Medium was supplemented using 10% fetal bovine serum, L glutamine, penicillin and streptomysin. 1% phytohaemaglutinin was added to stimulate cell division. Content was gently mixed and incubated at 37^{o}C and 5% CO_2 for twenty four (24) hours. At 24th hours, kolaviron and doxycycline were added to the test group and control group respectively then incubated for 48 hours. Cells were harvested after the total of 72 hours.

Treatment

Samples treated with kolaviron were the test group; those treated with doxycycline were the positive control group while the untreated samples were the control group. Final Concentration of Kolaviron used was 5µg/ml (Farombi *et al*., 2004) while that of doxycycline was 2µg/ml (Keiser *et al*., 2012).

3.2.5 *In vitro* Mitotic Index Assay

Principle: cell cultures are exposed to test substance, after predetermined exposure time, the culture is treated with metaphase arresting substance (colcemid), and cells are then harvested, stained and microscopically viewed.

In vitro mitotic Index was performed according to Ozkyul *et al*, 2005.

At the 24th hour of cell culture, 5µg/ml of kolaviron and 2µg/ml of doxycycline was added to their respective tubes with an extra tube serving as control (RPMI 1640 medium was added to normalize the condition). After shaking to mix, the content of the tubes was incubated at 37° C for 72hours. At the 70th hour, 0.1ml of colcemid solution (10µg/mL) was added to each tube and mixed by shaking gently. At the 72nd hour of incubation, cells were harvested by centrifugation followed by hypotonic treatment (0.075M KCl) and fixing on fresh fixative solution (methanol: acetic acid 3:1). The fixative step was repeated 3 times. Slides were air dried and stained with 5% Giemsa.

The mitotic index was calculated as the proportion of metaphase for 1000 cells as shown;

Percent mitotic index= number of metaphase × 100/total number of cells

3.2.6 RNA Isolation

Principle: Bioneer reagent works by maintaining RNA integrity during homogenization. Addition of chloroform, after centrifugation, separates the solution into aqueous and organic phases. RNA remains in aqueous phase which is precipitated using isopropanol.

Cells were harvested from culture by centrifugation for 5 minutes after which the supernatant was decanted leaving a little of the media with the cells. The tube containing the cells was then vortexed shortly. 200µl of the cell lysate was added to a 1.5 ml eppendorf tube. 400µl of Binding

buffer was added to the tube and mixed lightly by vortexing for five seconds to ensure efficient lysis. The tube was then left to incubate for 10 minutes at room temperature.

100µl of isopropanol was added and vortexed for 5 seconds, spun down for five seconds. The binding column was then fitted to the collection tube and the liquid was carefully transferred to the binding column. The column was centrifuged for 1 minute at 8000rpm until the liquid had completely passed through. The binding column was then transferred to a new collection tube.

500µl of wash buffer (W1) was added to the column and centrifuged at 8000rpm for 1 minute. The column was transferred to another collection tube 500µl of wash buffer 2 (W2) was added and centrifuged for 1 minute at 8000rpm then spun down again at 13000 rpm to remove residual alcohol. The binding column was transferred to a new collection tube and 50µl of elution buffer (pre heated at 60℃) was added and was allowed to stand for 1 minute, then centrifuged at 8000rpm for 1 minute. RNA was dissolved in 50µl of RNase-free water.

3.2.7 Quantification of RNA

Purified RNA was quantified using its absorbance at 260nm (A_{260}). A small amount of RNA was diluted (1µl of RNA to 9µl DEPC water i.e 1:10 dilution) for reading on the spectrophotometer.

RNA concentration was calculated using the equation:

RNA concentration (µg/mL) = A_{260}× 40 µg/ml × dilution factor

3.2.8 Complementary DNA Synthesis

Principle: using the polymerase chain reaction, template RNA is transcribed to a more stable cDNA. This requires the enzyme reverse transcriptase, primers, dNTPs.

The isolated RNA was transcribed into complementary DNA using the cDNA synthesis kit. The protocol was followed according to the manufacturer's instructions (Bioneer, Accuprep, USA) for the 20 μL containing 1µl of $dN_{12,}$ 1µl of template RNA and 18µl of DEPC water reverse transcription reaction mixtures. The mixture was incubated at 70C for 5 minutes and placed on ice, the incubated mixture was transferred to the premix tubes and filled up with DEPC water, the blue lyophilized pellet was dissolved by vortexing and briefly spun down, and mineral oil was added to each tube. The reaction condition was as follow: primer annealing at 30˚C for 5minutes, cDNA synthesis at 42˚C for 60 minutes and heat inactivation at 95˚C for 5 minutes.

3.2.9 Gene expression/Real-time PCR

Principle: Real-time PCR is carried out in a thermocycler with the capacity to illuminate beam of light and detect fluorescence. Involves the use of an intercalating dye whose fluorescence increases as more copies of gene is generated through cycles of polymerase chain reaction until the fluorescence is strong enough to be detected (threshold frequency).

Quantitative Real-Time Polymerase Chain Reaction (Q-PCR) was performed using a Light Cycler system (Version 3.5, Roche, Basel, Switzerland). Each sample was tested in duplicate and 18S ribosomal RNA was used as an internal control. Primers for VEGF-C, TLR2 and 18S ribosomal RNA are listed in Table 3.1.For the reaction, 5µL of reverse-transcribed products, 12µL ready-to use SYBR Green I Master mix, 1µL of RNAse free water and 1µL each of forward and reverse primers in 20µl final volume. Thermocycler conditions consisted of 1 cycle 95˚C for 15 min. Forty cycles, 95˚C for 15 s, 62˚C for 15 seconds, and 72˚C for 30s. A melting curve was obtained for each quantitative PCR run and the second derivative maximum method was used to determine the crossing point (Cp) for individual samples. The real-time PCR data

were analysed using the $2^{-\Delta\Delta CT}$ relative quantization method following the manufacturer's instructions.

Primers used for Gene expression

TLR2: Forward 5′GGGTCATCATCAGCCTCTCC-3′

Reverse 5′-AGGTCACTGTTGCTAATGTAGGTG-3′ (Sabroe *et al*., 2002)

VEGFC: Forward 5′CACGAGCTACCTCAGCAAGA- 3′

Reverse 5′-GCTGCCTGACACTGTGGTA-3′ (Leclers *et al*., 2006)

18S Ribosomal RNA: Forward 5′ CGGCTACCATCCAAGGAA 3′

Reverse 5′ GCTGGAATTACCGCGGCT 3′ (Lossos *et al*., 2003)

3.3 Data Analyses

To address biological variability, experiments were repeated three (3) times. Numerical results were presented as Mean ± Standard Deviation. Variability between groups was measured using one way ANOVA using Statistical Package for Social Sciences Software (SPSS) version 20.0 (SPSS Inc., Chicago, Illinois, USA). Level of significance was measured using least significant difference (LSD). $P<0.05$ were considered significant. Correlation analysis was done using Microsoft excel.

CHAPTER FOUR

4.0 RESULTS

4.1 Anti-proliferative effect of kolaviron on the cultured peripheral blood lymphocytes of *Wuchereria bancrofti* infected blood using Mitotic index assay

The anti-proliferative effect of kolaviron and doxycycline were analysed using the mitotic index assay as shown in Figure 4.1. The result shows a significant ($p<0.05$) decrease in mitotic index of the treatment groups as compared to the untreated, however, doxycycline treated samples showed a slightly greater effect although not significantly different from the kolaviron treated group.

4.2 The effect of kolaviron on the expression of TLR2

Expression of toll like receptor 2 (Figure 4.2) was significantly ($p < 0.05$) reduced in the kolaviron treated group and doxycycline treated group when compared with of that the untreated control in the concentration used, with kolaviron having more pronounced effect.

4.3 The effect of kolaviron on the expression of VEGF-C

A significant ($p < 0.05$) decrease on the expression of vascular endothelial growth factor c (Figure 4.3) was observed in the kolaviron treated and doxycycline treated group when compared to the control group although more pronounced effect was observed in the kolaviron treated group when compared to that of doxycycline.

4.4 Correlations between mitotic index, VEGF-C and TLR2 expression levels

Correlation studies showed positive correlation between mitotic index, toll like receptor 2 and vascular endothelial growth factor C expression levels. There was a weak positive correlation between mitotic index and Toll like receptor 2 while the correlation between TLR2 and VEGFC was strongly positive as shown in Figure 4.4.

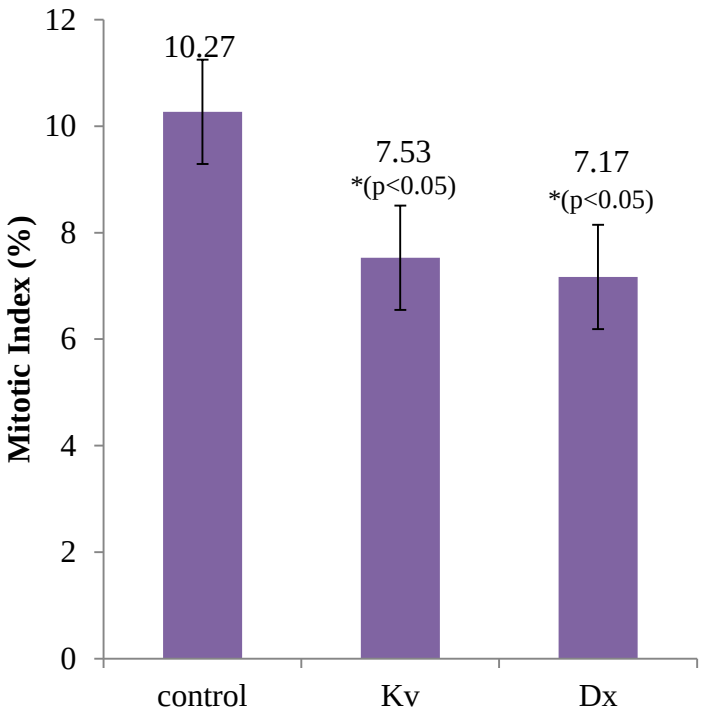


Figure 4.1: Mitotic index in untreated, kolaviron and doxycycline treated samples

Where n=6 and Values with* significantly different from control at *P<0.05*.

Key;
Control- untreated sample as negative control
KV- kolaviron treated sample as test sample (5µg/ml)
Dx- doxycycline treated sample as positive control (2µg/ml)

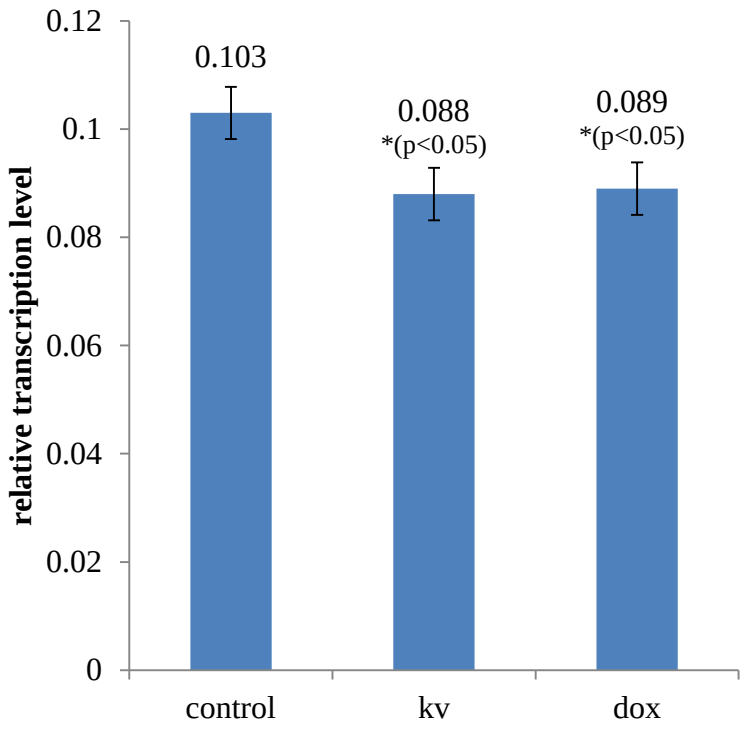


Figure 4.2: Expression of TLR2 in untreated, kolaviron and doxycycline treated samples

Where n=6 and Values with* significantly different from control at *P<0.05*.

Key;
Control- untreated sample as negative control
KV- kolaviron treated sample as test sample (5µg/ml)
Dx- doxycycline treated sample as positive control (2µg/ml)

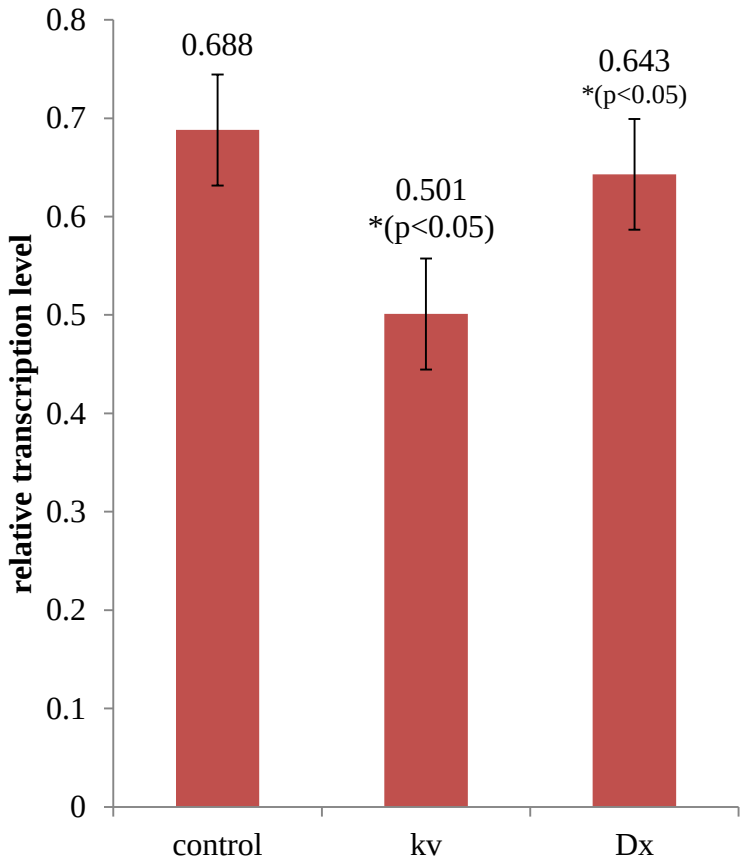


Figure 4.3: Expression of VEGF-C in untreated, kolaviron and doxycycline treated samples

Where n=6 and Values with*significantly different from control at *P<0.05*.

Key;
Control- untreated sample as negative control
KV- kolaviron treated sample as test sample (5µg/ml)
Dx- doxycycline treated sample as positive control (2µg/ml)

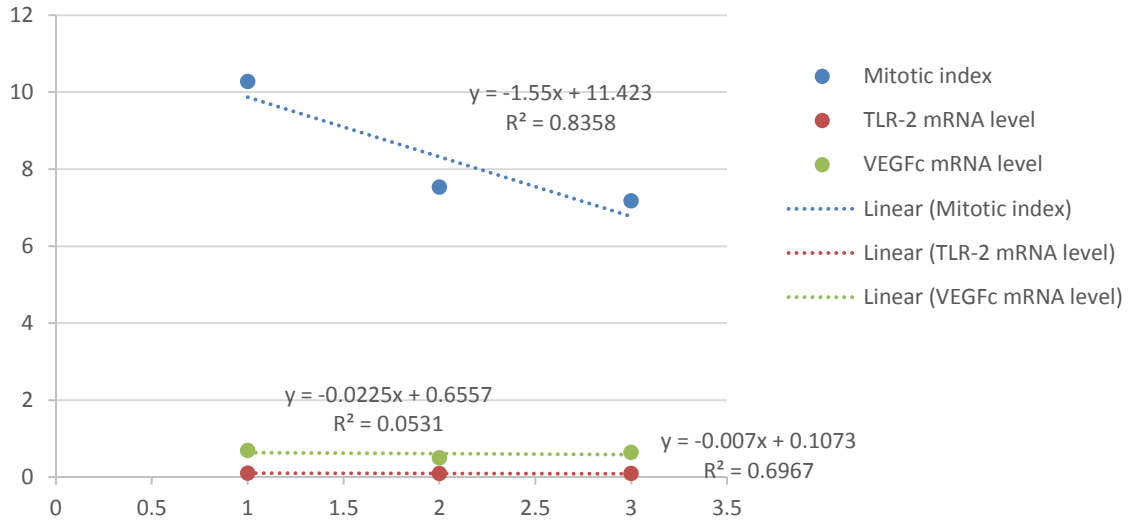
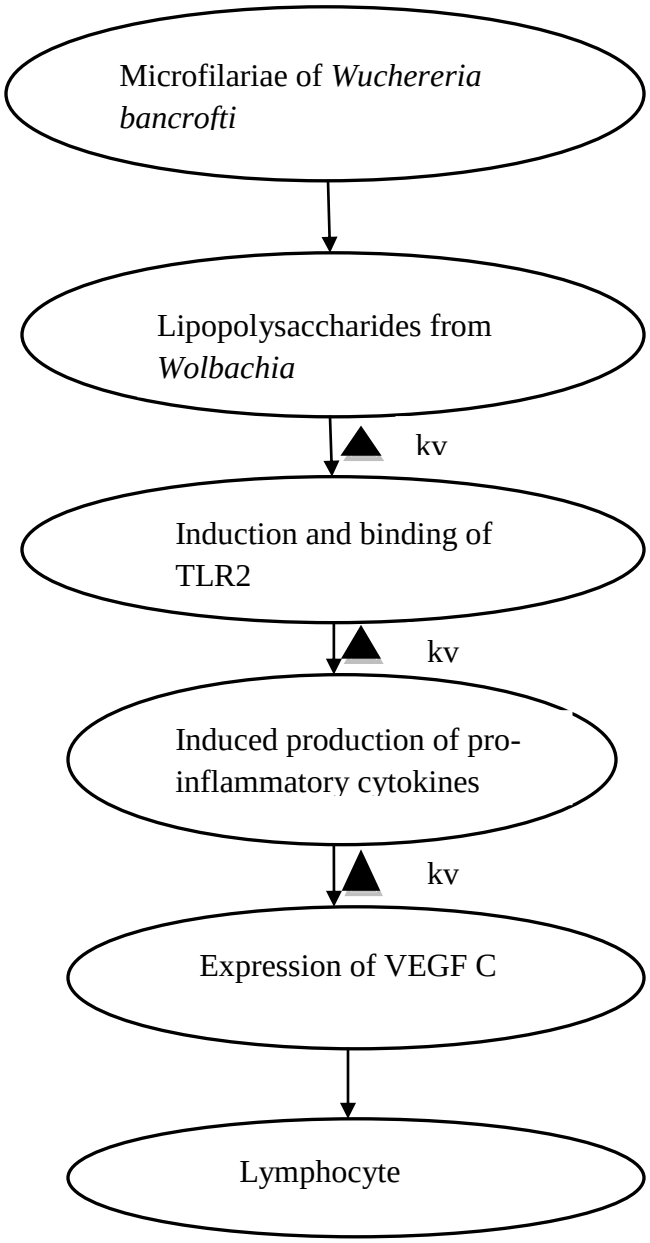


Figure 4.4: Correlation plot between Mitotic index, VEGF-C expression levels and TLR-2 expression levels


Microfilariae of *Wuchereria bancrofti*
Lipopolysaccharides from *Wolbachia*
kv
Induction and binding of TLR2
kv
Induced production of pro-inflammatory cytokines
kv
Expression of VEGF C
Lymphocyte

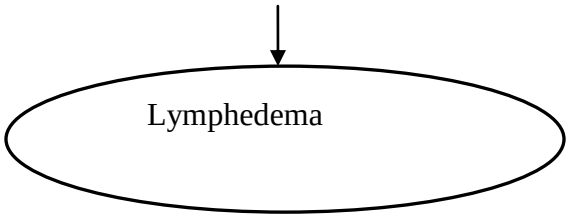


Figure 4.5: Proposed Mechanism of action of Kolaviron in VEGFC and TLR2 Expression.
Key:
▲Kv- Possible point of Kolaviron inhibition

CHAPTER FIVE

5.0 DISCUSSION

Lymphatic filariasis is caused by *W. bancrofti* which led to severe damage and painful swelling. Over time, major advances in the management of lymphatic filariasis has given the potential of eradicating the disease with the use of antifilarial drugs (diethylcarbamazine, ivermectin and albendazole) (Debrah *et a*l., 2006), however, these drugs have limited microfilaricidal effect and do not improve the pathology associated with lymphatic filarasis (Hoerauf *et al*., 2011). In this study, we report for the first time, the anti- proliferative effect of kolaviron and down regulation of vascular endothelial growth factor C and toll like receptor 2 in *W. bancrofti* infected blood lymphocytes, thus, possible role of kolaviron in ameliorating the pathology of lymphatic filariasis. In treatment of lymphatic filariasis, doxycycline was observed to reduce plasma levels of VEGF-C and its receptor VEGF3 as well as lymphatic dilation upon treatment of patients with 200mg/day of doxycycline for 6 weeks (Debrah *et al*., 2006) as such was used as the positive control in this study.

Mitotic index measures the proportion of cells in the metaphase phase of the cell cycle and when inhibited, it can be considered as cell death or delay in proliferation kinetics of cells (Portman *et al*., 2012) and studies have suggested that active proliferation is a feature of lymphatic filariasis (Bennuru and Nutman, 2009). In this study, Kolaviron and doxycycline significantly reduced the proliferation of cells in the lymphatic filariasis cultured blood lymphocytes as compared to those of the control, however, reduction in proliferation was more evident in the doxycycline treated samples further confirming the anti-proliferative effects of both as reported by (Ayepola *et al*., 2014; Sekeroglu *et al*., 2012). Kolaviron may have acted by causing delay in proliferation.

W. bancrofti and other filarial worm houses a Gram negative bacteria called *Wolbachia*. Wolbachia modulates the host immune system by signaling through adaptor proteins (Brattig et al., 2004). This immuno modulation is not restricted to Wolbachia alone as lymphatic filariasis is characterised by secondary bacterial infection (Babu *et al*., 2012). This signaling is done using toll like receptor 2 (Hise *et al*., 2007, Turner *et al*., 2009).

In this study, results showed increased expression of toll like recptor 2 in the untreated samples but down regulation were observed upon treatment with kolaviron and doxycycline with kolaviron having more pronounced effect *in vitro.* This reduction in down regulation may have been due to anti microbial effect of both doxycycline and kolaviron (Farombi and Owoeye, 2011), thus, preventing the interaction of *Wolbachia* and other secondary bacteria from signaling through toll like receptors. New treatments involving TLR modulation could potentially provide new avenue in ameliorating pathology in lymphatic filariasis (Babu *et al*., 2012).

Filarial pathology is characterized by high levels of immune complexes (Senbagavalli *et al*., 2011). A potential mechanism by which pro-inflammatory cytokines could lead to lymphatic damage is through ability to induce production of lymphangiogenic and angiogenic factors. Vascular endothelial growth factor is an angiogenic factor with vascular permeability and angiogenic effects and plays a central role in vasculogenesis, neoangiogenesis by promoting survival, migration and proliferation of endothelial cells (Takahashi and Shibya, 2005). The binding of VEGF-C and its soluble receptor is important for lymphatic proliferation and it is believed to be involved in lymphatic dilatation and lymphedema (Debrah *et al*., 2007). The current study showed high expression of vascular endothelial growth factor C in the untreated control group when compared to that of the treatment groups hence, the treatments help to down regulate the expression of VEGF-C *in vitro*. Report showed that doxycycline inhibits mettalloproteinases responsible for breakdown of connective tissues and inhibit VEGFs (Monk *et al*., 2011). Kolaviron may have also inhibited VEGF expression although the mechanism of doing this is yet to be studied/ reported.

TLR ligands and filarial antigen were shown to induce higher expression/production of VEGF-A, VEGF-C and angiopoetin 1 in those with chronic pathology suggesting an association between pattern recognition pathway signaling and lymphangiogenesis (Anuradha *et al*., 2012; Hise *et al.*, 2007). Correlation studies showed strong positive correlation between VEGFC and TLR2, this, confirming an association between TLR2 and VEGFC.

The was also positive correlation between the level of proliferation and expression of vascular endothelial growth factor C and toll like receptor 2 in lymphatic filariasis. Vascular endothelial

growth factors are potent mitogens which are highly specific for endothelial cells hence promoting angiogenesis and lymphangiogenesis (Hoeben *et al*., 2004).

Proposed mechanism of action of Kolaviron in this study was at three points. Kolaviron having shown to have anti microbial effect may have inhibited the binding of *Wolbachia* and other secondary microbes from binding to the TLR2 adapter protein (Sabroe *et al*., 2002) hence no signaling/induction of pro inflammatory cytokines and angiogenic factors.

Kolaviron may have also inhibit the cascade of reactions leading to the production of pro inflammatory cytokines or the expression of angiogenic and lymphangiogenic factors

Inhibition of VEGF-VEGFR is gaining importance in the fields of tumour immunology, vascular disease and other inflammatory disorders (Carmeliet and Jain, 2011). Prolonged treatment with flavonoids has been considered to be effective in reducing lymphedema (Shenoy, 2008), which invariably might have supported our findings with regards to the observed effects of kolaviron, a flavonoid from *Garcinia kola*.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION, RECOMMENDATIONS

6.1 SUMMARY

i. Kolaviron was shown to reduce the mitotic index of cells in cultured peripheral blood lymphocytes of lymphatic filriasis patients hence may be anti proliferative

ii. Kolaviron significantly reduced the level of expression of toll like receptor 2 and vascular endothelial growth factor C.

iii. There was positive correlation between the rate of proliferation (mitotic index), expression of TLR2 and VEGF-C.

iv. There was also positive correlation between the expression of TLR2 and VEGF-C.

6.2 Conclusion

Kolaviron rich portion of *Garcinia kola* exhibited anti-proliferative effect and down regulation of vascular endothelial growth factor C and toll like receptor 2 in *W. bancrofti* infected blood lymphocytes. The findings from this work might have unraveled the potency of kolaviron in the management of complications associated with lymphatic filariasis.

6.3 Recommendation

In vivo studies on the effect of kolaviron in managing lymphatic filariasis also should be done so as to ascertain its effect on other symptoms in the disease state.

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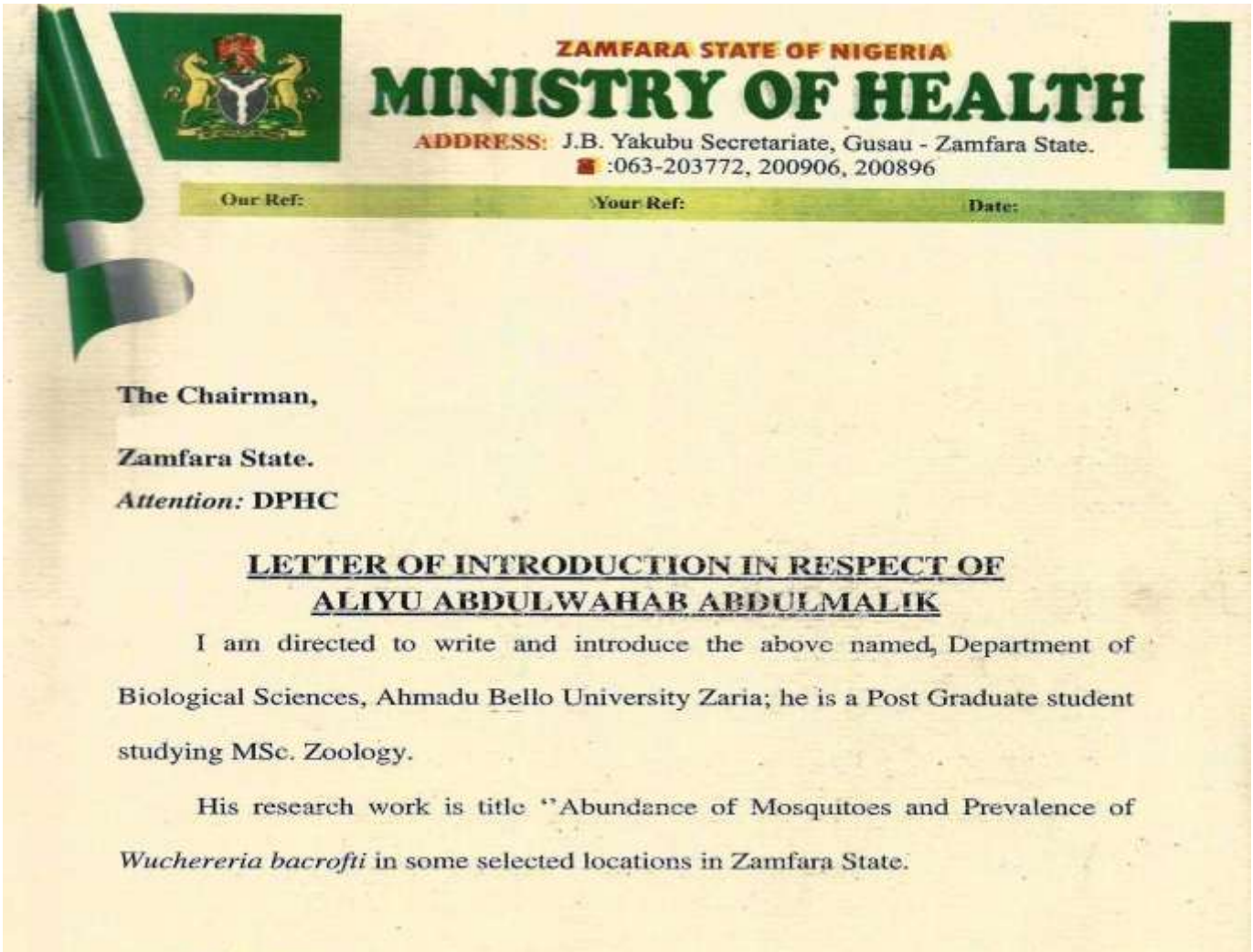
APPENDIX I



ZAMFARA STATE OF NIGERIA

MINISTRY OF HEALTH

ADDRESS: J.B. Yakubu Secretariate, Gusau - Zamfara State.
:063-203772, 200906, 200896

Our Ref: Your Ref: Date:

The Chairman,

Zamfara State.

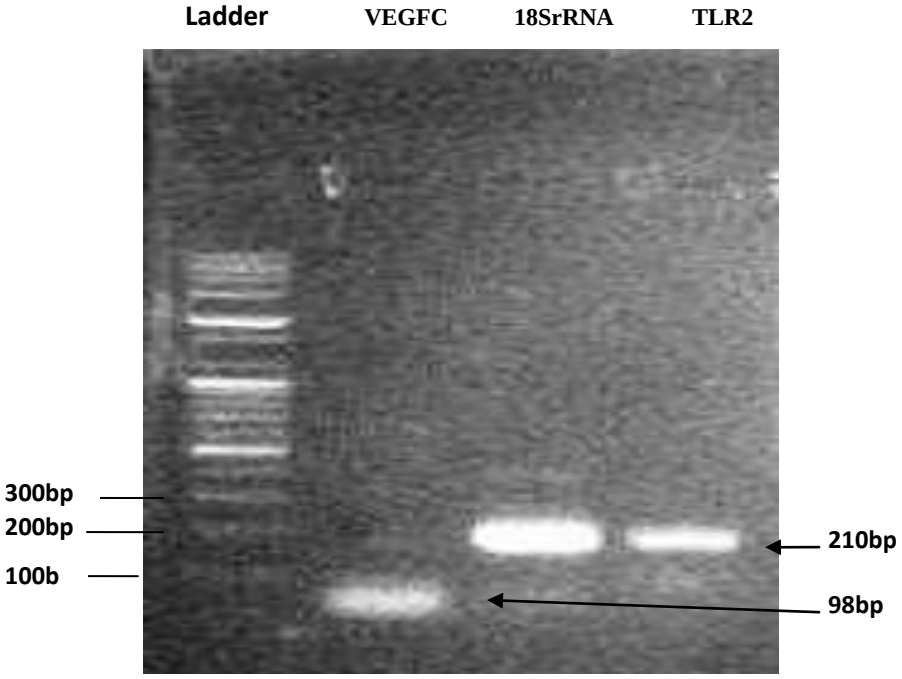
Attention: **DPHC**

LETTER OF INTRODUCTION IN RESPECT OF ALIYU ABDULWAHAB ABDULMALIK

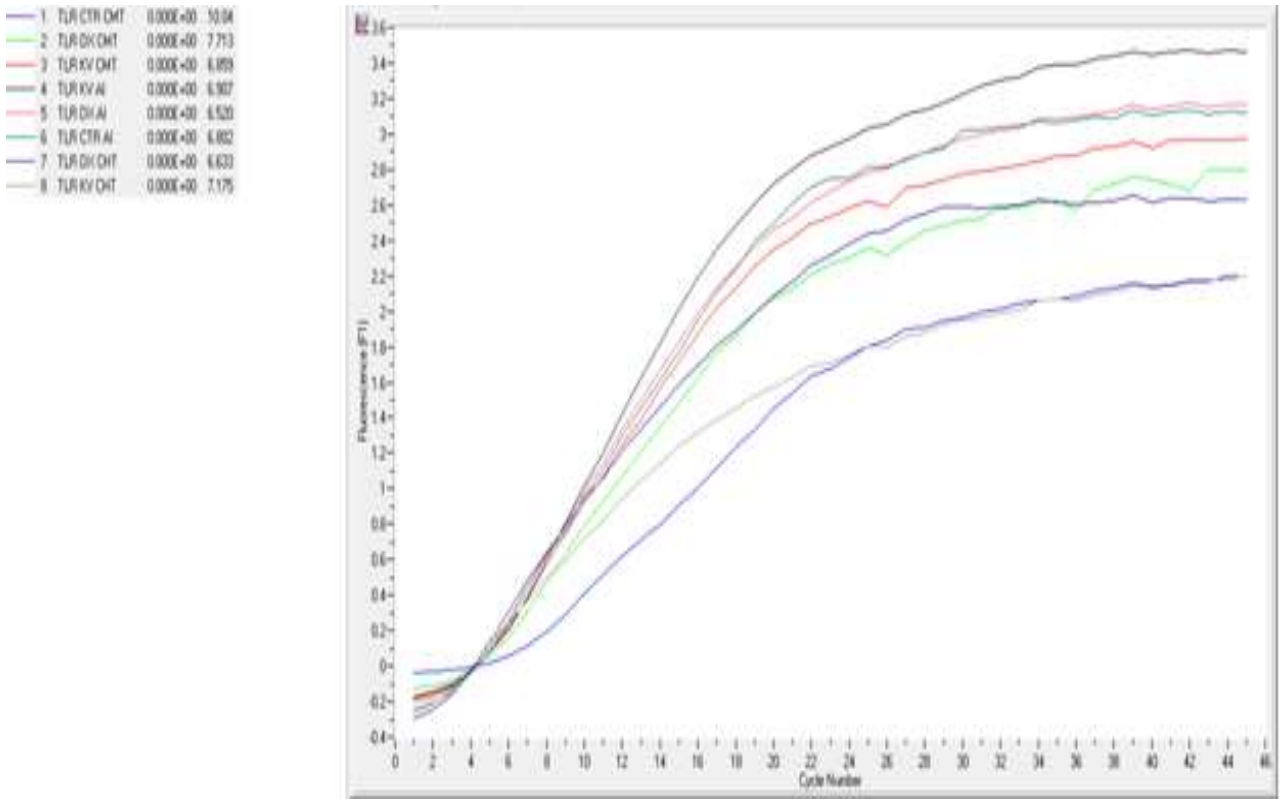
I am directed to write and introduce the above named, Department of Biological Sciences, Ahmadu Bello University Zaria; he is a Post Graduate student studying MSc. Zoology.

His research work is title ''Abundance of Mosquitoes and Prevalence of *Wuchereria bacrofti* in some selected locations in Zamfara State.

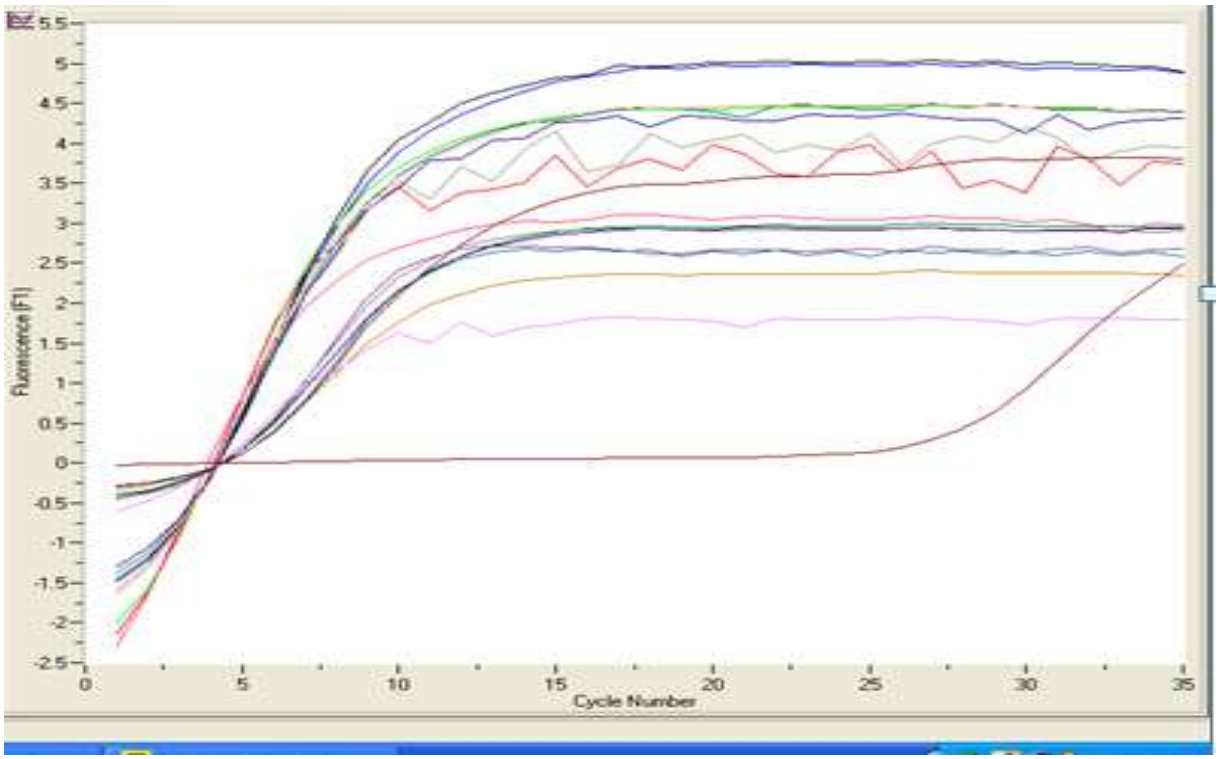
Ethical clearance. Sample collection was done in collaboration with Abdulwahab Abdulmalik an MSc. student (supervised by Prof. I.S Ndams).



Agarose gel picture showing band of VEGFC, 18SrRNA and TLR2 gene primers after PCR optimization



Amplification plot for TLR2 gene expression



Amplification plots for VEGFc and 18S ribosomal RNA

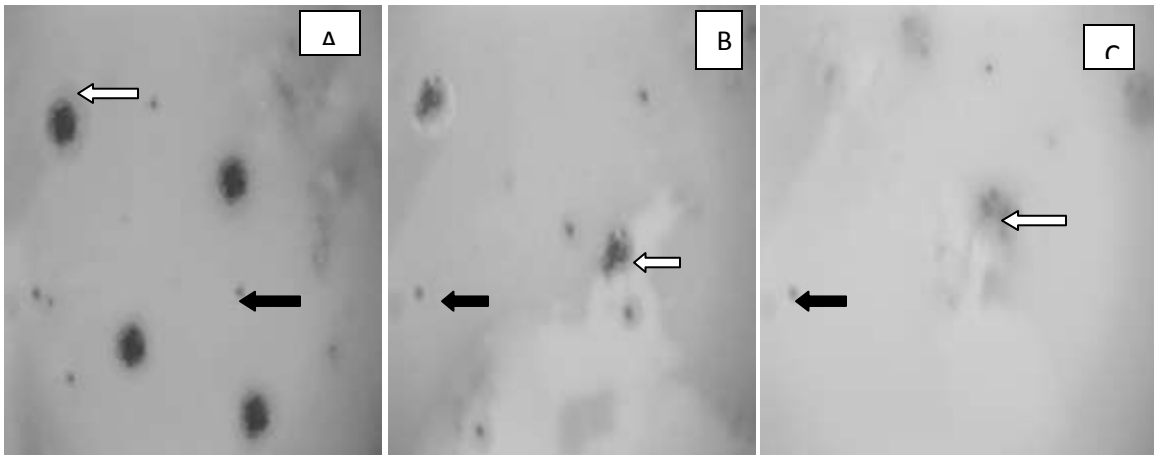


Plate showing mitotic index: A control, B(Doxycycline treated 2µg/ml), C(kolaviron treated 5µg/ml) with black arrow showing cells at interphase and white arrow showing cells at metaphase using the mitotic index assay (×20 magnification, Giemsa stain)