SUSCEPTIBILITY AND BEHAVIOURAL RESPONSES OF THE GENUS *ANOPHELES* (DIPTERA: CULICIDAE) TO BEDNETS IMPREGNATED WITH A SYNERGIZED PYRETHRUM INSECTICIDE FORMULATION AND POTENTIAL FOR MALARIA CONTROL

BY

KEFA STANLEY ORWA SUM

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DEPARTMENT OF ZOOLOGY

MASENO UNIVERSITY

ABSTRACT

The use of insecticide-treated nets (ITNs) has become a key cost-effective intervention in malaria control. However, there is need to develop new insecticide formulations to sustain the technology and manage the threat of *Anopheles* mosquito resistance to pyrethroid insecticides solely used in ITNs. Susceptibility and behavioural responses of *Anopheles gambiae* s.l. to different bednet fabrics treated with synergized natural pyrethrum-formulation was assessed using WHO cone and tunnel bioassay procedures. Effect on indoor resting and feeding behaviour was evaluated under field situation in 24 selected village huts in Ahero area. *Anopheles* siblings were identified using polymerase chain reaction (PCR) while real time PCR (RT-PCR) was used to genotype *kdr* gene mutation on species obtained from Kisian, Ahero and Kipsitet areas. Data were transformed into logarithms or arcsine and subjected to analysis of variance (ANOVA). Means were compared using least significant difference (LSD, P=0.05) while logit or regression analysis was used to model dose-response relationships. Results showed no significant additional effect on knockdown at durations longer than 15 minutes post-initial exposure to treated nets regardless of the dose (KD-15 min and KD-30, P= 0.6312; KD-15 and KD-60min, P=0.1590). After 1-3 washes, nets impregnated with natural pyrethrum-formulation at 500 mg/m^2 still achieved more than 85% knockdown and mortality. Unwashed nets had 90% feeding inhibition and over 90% knockdown and kill up to six months. There was a significant (P=0.0001) fabric-dose interaction where nylon achieved standard 80% mortality on mosquitoes at 200 mg/m^2 as compared to 375 mg/m^2 with polyester. It was however, not possible to achieve 80% kill with cotton at the test doses. No *kdr* genes were detected in *An. arabiensis* but there was 100% frequency of the L1014S *kdr* mutation in the *An. gambiae* s.s. Natural pyrethrum formulation achieved significantly (P=0.0001) greater kill than pyrethroids against *An. gambiae* s.s. with *kdr* genes. There were significantly (P=0.0001) higher numbers of indoor resting and fed *An. gambiae* s.l. collected in houses with untreated nets than houses with bednets treated with natural pyrethrum-formulation. Overall, the results demonstrate that knockdown (KD) tests on *Anopheles* mosquitoes could be standardized at 15 minutes post-initial exposure and not 60 minutes, as previously reported, to save on time and resources. The results give empirical evidence and parameters pertinent to effective use of natural pyrethrum formulation in ITNs. Susceptibility of *An. gambiae* s.s. with *kdr* alleles and wild forms to the formulation provides crucial lead to management of resistance in malaria vectors.

CHAPTER ONE

1.0 INTRODUCTION

In tropical and sub-tropical countries of the world, malaria is still a major problem and one of the principal causes of human morbidity and mortality (WHO, 1996; Greenwood and Mutabingwa, 2002; Pages *et al.*, 2010). The disease kills over 1 million people worldwide annually and about 90% of the deaths occur in Africa with 70% of the cases being children under the age of five, especially in sub-Saharan Africa (WHO, 1996; Breman, 2001; Snow *et al.*, 2004). Other vulnerable groups include pregnant women and populations with low inherent immunity (WHO, 1996). In Kenya, about 70% of the population is regularly exposed to the most deadly malaria parasite, *Plasmodium falciparum*, infection. The disease accounts for 35% of the outpatient cases and 13.4% of mortality at national level (MOH, 2010). In the endemic zones where malaria is transmitted all year round, it accounts for upto 50% of outpatient morbidity and also acts as a leading cause of hospital admission accounting for about 23% of all inpatient cases (MOH, 2010).

Anopheles s.l. mosquitoes are the principal vectors of malaria transmission in the tropical and sub-tropical areas of the world (WHO, 1986). The species have a worldwide distribution, occurring in both tropical and temperate regions (Service, 1980). There are over five hundred known species of *Anopheles*, but only sixty are known to transmit malaria (Service, 1980), The remarkably high transmission rates in sub-Saharan Africa reflect the particular capacity of Africa's main vector mosquitoes, the *Anopheles gambiae* complex of species with their high tendency towards human biting (Sachs and Malaney, 2002).

The six species complex, are members that are difficult and sometimes impossible to distinguish morphologically (Coluzzi, 1984; Gillies and Coetzee, 1987; Fontenille and Lochoven, 1999). They are named *An. gambiae* sensu strictu, *An. arabiensis*, *An. merus*, *An. melas*, *An. quadriannulatus* and *An. bwambae*. The complex that is collectively known as *Anopheles gambiae* s.l. varies in their ability to transmit malaria (White, 1974; Hunt *et al.*, 1998) . The four kinds of malaria parasites that have been known to infect humans are *Plasmodium falciparum, P. vivax, P. ovale or P. malariae.* (Schlagenhauf, 2004) but lately, *P. knowlesi* has also been recognized to infect human (Cox-Singh *et al.*, 2010). Of these, *P. falciparum* causes a large majority of the clinical cases and is responsible for about 80% of infections and 90% of deaths (Bozdech *et al.*, 2003). Despite the strong effort to eradicate malaria, the disease burden is still on the rise. Estimates indicate that, the number of cases could double in the next twenty years if there is no development of new methods for its control (Sachs and Malaney, 2002).

The spread of malaria in many developing countries in tropical and sub-tropical zones adversely affect health and socio-economic development (Sachs and Malaney, 2002). These facts, taken together, have increasingly neccesitated the need for co-ordinated effort to prevent further deterioration of the situation through development of innovative and novel products aimed at control of mosquito vectors.

Malaria control encompasses a variety of measures that may protect individuals against infections or development of the disease. One of the ways to protect people from the parasite is through chemo-prophylaxis using anti-malarial drugs (WHO, 1997; NIAID, 2000). Other approaches for preventing malaria are to reduce the incidence of bites and

population of *Anopheles* mosquitoes through the use of chemicals or biological control agents (Greenwood, 2008; Mendis *et al.*, 2009).

Early efforts to control malaria in the 1950s and 1960s by use of DDT and other insecticides by indoor residual spraying (IRS) to control adult mosquitoes was the first significant progress in malaria eradication through vector control (Gramiccia and Beales, 1988; Mabaso *et al.*, 2004). However, due to health and environmental concerns, the use of DDT was restricted and even banned in some countries (UNEP, 2001). DDT also suffered a major setback due to development of resistance by many insect species (Curtis, 1962; Kasap *et al.*, 2000). Lately, other technologies especially use of insecticide-treated nets (ITNs) have become promising tools against malaria vectors (Philips-Howard, 1998; Lengeler, 2004). ITNs can be effectively used together with other methods like IRS, source reduction through larviciding and combination drug therapy in malaria control (Greenwood, 2008). However, the success of vector control is threatened by the development of resistance to insecticides in the market by *Anopheles* mosquitoes leading to continued upsurge in malaria cases (WHO, 1986; Chandre *et al.*, 1999; Vulule *et al.*, 1999; Hargreaves *et al.*, 2000; Ranson *et al.*, 2000).

At present, pyrethroid insecticides are the only option for impregnating bednets for malaria control. However, resistance to this group of insecticide has been reported in many African countries including Burkina Faso, Cote d' Ivoire (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999; Diabate *et al.*, 2004), Ghana (Adasi *et al.*, 2000), Kenya (Vulule *et al.*, 1999; Ranson *et al.*, 2000; Stump *et al.*, 2004) and Malawi (Hunt *et al.*, 2010). This may hamper the effective use of these treated bednets in areas where enhanced tolerance or resistance to pyrethroids has been reported (Curtis *et al.*, 1990;

Curtis *et al.*, 1996; Yewhalaw *et al.*, 2011). The solution to sustained use of insecticide-impregnated bednets could, therefore, be in the consideration of alternative insecticide molecules or products (Zaim and Guillet, 2002) including the use of safer bednet treatment insecticide formulation based on natural pyrethrum, *Chrysanthemum cinerariaefolium,* a perennial temperate plant with small white daisy-like flowers. Despite their photolability, when suitably formulated with a synergist and anti-oxidant, these natural pyrethrins increase in their stability and residual efficacy (Sattelle and Yamamoto, 1988; Warui and Mutinga, 1994).

Currently, there is scanty documented evidence on the use of pyrethrum in ITNs despite it being a natural botanical insecticide that is environmentally friendly with several advantages over chemically synthesized insecticides (www.bugfreebackyards.com). It is against this background that the current study evaluated the use of natural pyrethrum-formulation in treatment of bednets.

Effective use of an insecticide formulation in ITNs calls for an initial understanding of the influence of basic parameters including dose-response effects that help to formulate field application requirements, structure-activity correlation and resistance measurements (Frommer *et al.*, 1983; Moyses and Gfeller, 2001). Further knowledge on the effect of treated bednets on successful feeding is important because the irritant effect of certain insecticides can reduce mosquito human biting habit in houses and divert mosquitoes to feed on domestic animals sheltered near dwellings of human (Mathenge *et al.*, 2001; Killeen *et al.*, 2007). There is, however, scanty information on these parameters with respect to use of natural pyrethrum formulation in ITNs.

Washing of a net eventually reduces the insecticide dose to a level with minimal entomological impact (Lindsay *et al.*, 1991; Ordonez-Gonzalez *et al.*, 2002). However earlier studies have shown that insecticide active ingredients responded differently to washing (Rafinejad *et al.*, 2008). No study currently shows efficacy and wash resistance of nets treated with a natural pyrethrum formulation and this was addressed in the current study. Studies elsewhere, have shown that differences in bioefficacy of insecticide-treated nets may closely be linked to the nature of fabrics (Rozendaal, 1989; WHO, 2006; Rafinejad *et al.*, 2008), and specific insecticide formulation (WHO, 1986; Lines *et al.*, 1987; Hossain *et al.*, 1989). However, there is little information on the influence of the type of netting fabric and varying impregnation dosage on bio-efficacy of natural pyrethrins formulation-treated bednets.

Emergence of insecticide resistance is one of the key threats to successful use of ITNs (Vulule *et al.*, 1999; Ranson *et al.*, 2000). The underlying resistance mechanisms in *Anopheles* s.l. to pyrethroids and pyrethrins is mainly associated with target site insensitivity arising from single point mutation in the sodium channel gene, often referred to as knockdown resistance (*kdr*) (Scott and Matsumura, 1981; Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). One strategy to fight resistance is to incorporate synergists in an insecticide formulation to effect competitive metabolic enzyme inhibition, (PBK, 1987; Ramoutar *et al.*, 2009), however, their specific role in managing *kdr* resistance is unclear. There was need therefore, to evaluate the effect of synergized natural pyrethrum formulation on mosquitoes with *kdr* genes and also monitor the occurrence of *kdr* mutation within the mosquito populations to generate information relevant to the overall vector control strategy.

Work under laboratory conditions has demonstrated the effectiveness of natural pyrethrum formulation-treated nets against laboratory bred *An. gambiae* s.s. (Sum *et al.*, 2011). Other studies have shown varied responses of different vector mosquitoes to insecticides due to their specific feeding and resting behavioural patterns which may greatly influence their reaction to specific active ingredients in the ITN (Jawara *et al.*, 1998; Mosha *et al.*, 2008). Despite the demonstrated potential for use of natural pyrethrum formulation in ITNs, to date there has not been an evaluation of the performance of nets treated with this product against common malaria vectors under field situation.

1.1 Statement of the problem

Insecticide-treated net (ITN) is an effective technology in malaria control. Trials undertaken in many African countries have shown remarkable success in its reduction of overall mortality and morbidity (Alonso *et al.*, 1991; Lines, 1996; Gimnig *et al.*, 2003; terKuile *et al.*, 2003). Currently, the WHO pesticides evaluation scheme (WHOPES) recommends only the pyrethroids as the active ingredient (a.i) used in insecticide formulations for treatment of bednets (Zaim *et al.*, 2000). However, emerging resistance to pyrethroid insecticides by *Anopheles* malaria vectors threatens to reduce the potency of this important control method (WHO, 1986; Elisa *et al.*, 1993; Chandre *et al.*, 1999; Vulule *et al.*, 1999; Ranson *et al.*, 2000). Further emphasis on the use of pyrethroids in long life nets (LLNs) with residual protection of over 3 years could select for resistance in vector mosquitoes. The possibility that prolonged insecticide use could lead to behavioral changes that are advantageous to the target population has been recognized (WHO, 1986). Some pyrethroids particularly most alpha-cyano substituted compounds have also been observed to

cause sensation on the skin or mucosa (Rozendaal, 1989). Studies elsewhere, have also shown that bio-efficacy of insecticide-treated nets may closely be related to the nature of fabrics, among other properties (Rozendaal, 1989; WHO, 2006; Rafinejad *et al.*, 2008), with the possible link between specific insecticide formulation and the empirical bio-efficacy of ITNs (Lines *et al.*, 1987; Hossain *et al.*, 1989; Vatandoost *et al.*, 2006). The influence of the type of netting fabric and varying impregnation dosage on bio-efficacy of an insecticide is an essential step in determining field treatment rates and gives numerical measure of its effectiveness (Frommer *et al.*, 1983).

Successful feeding by a vector on a host is the most important component in disease transmission and is used in evaluating impact of vector control measures (Garret-Johnes and Shidrawi, 1969). The irritant effect of certain insecticides can reduce mosquito human-biting habit in houses and divert mosquitoes to feed on domestic animals or may enhance the potency of a treated net in case of tear or wear (Killeen *et al.*, 2007).

Washing of a net directly reduces the insecticide dose to a level with minimal entomological impact (Lindsay *et al.*, 1991; Ordonez-Gonzalez *et al.*, 2002). Earlier studies have also shown that insecticide active ingredients responded differently to washing (Rafinejad *et al.*, 2008). These scenarios therefore give rise to challenges in developing alternative products such as the natural pyrethrum formulation that is safe, effective and fast-acting when properly formulated (Sattelle and Yamamoto, 1988; Silcox and Roth, 1995) to sustain the technology by replacement or rotational use with the existing insecticides to manage resistance. The fact that the natural pyrethrum formulation may have a different profile from synthetic insecticides in

terms of selection of resistance and cross-resistance pattern may be of great value in controlling pyrethroid resistant vector mosquitoes. A natural pyrethrum formulation was successfully used in impregnation of wall cloths (Warui and Mutinga, 1994) and other fabrics (Bry *et al.*, 1977), though its efficacy on mosquito nets has not yet been documented.

Further detailed studies on the various aspects to understand the relationship between the mode of action of the chemical and the physical components of impregnated nets and behavioural responses of the vector could lead to substantive improvement on the use of ITNs in malaria control. As such, the current study evaluated and quantified the susceptibility in terms of knockdown and kill and behavioural responses (feeding, repellency) by exposing *Anopheles* malaria vectors to bed nets treated with a novel stabilized and synergized natural pyrethrum formulation.

1.2 Research questions

1. What is the effect of different doses of the natural pyrethrum-formulation impregnated on bed nets on knockdown and kill of *An. gambiae* s.s. and what is the optimum dose that achieves KD_{95}, LC_{80} for use in net treatment?
2. How does the nature of fabric and colour of a netting material influence the bio-efficacy of bed nets treated with natural pyrethrum-formulation against *An. gambiae* s.s?
3. What are the effects of repeated washing of netting treated with natural pyrethrum formulation on bio-efficacy against *An. gambiae* s.s., rate of degradation of pyrethrins and loss in bio-efficacy within six months under unwashed conditions?

4. What is the distribution of knockdown resistance (*kdr*) genes in *Anopheles gambiae s.l.* in Kipsitet, Ahero and Kisian areas and effect of natural pyrethrum formulation on wild and pink eyed *An. gambiae* s.s. with fixed *kdr* resistance genes?
5. What are the effects of pyrethrum formulation-impregnated nets on the behaviour (feeding, irritancy, and repellency), density and mortality of *An. gambiae* s.l. under laboratory and small-scale field situations?

1.3 Objectives of the study

1.3.1 The broad objective

To evaluate and quantify the susceptibility and behavioural response of *Anopheles* malaria vectors to bednets treated with a novel stabilized and synergized natural pyrethum formulation.

1.3.2 Specific Objectives

1. To determine the effect of different net impregnation doses of the natural pyrethrum formulation on knockdown and kill of *An. gambiae* s.s. mosquitoes and generate dose–response relationship, median lethal doses (KD_{50}, KD_{95}, LC_{50} and LC_{95}) and optimal net treatment dosage.

1. To assess the effect of different physical parameters including type of netting fabrics and colour on bio-efficacy of natural pyrethrum formulation-treated nets against *An. gambiae* s.s.
2. To determine the effect of repeated washing and six months persistence of netting treated with natural pyrethrum formulation in relation to bio-efficacy against *An. gambiae* s.s.

3. To determine susceptibility and distribution of knockdown resistance (*kdr*) genes in *Anopheles gambiae s.l.* in Kipsitet, Ahero and Kisian areas and effect of natural pyrethrum formulation on pink eyed *An. gambiae* s.s. with fixed *kdr* resistance genes
4. To evaluate the effect of pyrethrum formulation-impregnated nets on the behaviour (feeding, irritancy, and repellency), density and mortality of *An. gambiae* s.l. under laboratory and small-scale field situations.

1.4 Justification of the study

Pyrethrum crop is grown in Kenya, Tanzania, Rwanda, Democratic Republic of Congo (DRC), Papua New Guinea and lately in Tasmania (Australia) (Acland, 1975). However, Kenya has been the largest producer accounting for about 70% of world production with a potential to produce over 10,000MT of flowers annually (PBK, unpublished results) and this was also reported previously (Acland, 1975). The potential for local availabilty of natural pyrethrum in large quantities provides reason to conduct research into its suitabilty for use in ITNs as a local solution to the malaria menace.

Despite elaborate literature on the health impact of ITNs (Alonso *et al.*, 1991;Beach *et al.*, 1993; Gimnig *et al.*, 2003), there is not a corresponding effort in research into alternative insecticide formulations for use in ITNs. Besides, WHOPES recommends only pyrethroids as the active ingredient (a.i) for use in insecticide formulations for treatment of bed nets (Zaim *et al.*, 2000), thus validating the need for the current study. The use of the natural pyrethrum formulation in ITNs could be of benefit to millions at risk of malaria, a disease that costs Africa in excess of US $ 12 billion

annually leading to slowed economic growth of 1.3% (WHO, 2003). The fact that the natural pyrethrum formulation has shown significant efficay against resistant mosquitoes could be of great value in controlling pyrethroid resistant vector mosquitoes, a phenomenom that is threatening the success of ITNs. Besides, understanding the relationship between the mode of action of the chemical and the physical characteristiscs of impregnated nets and behavioural responses of the vector could lead to substantive improvement on the use of ITNs in malaria control. Apart from its contribution in tackling the grave malaria problem, pyrethrum crop brings income to many pyrthrum farmers and their dependants. Being a natural product, it is environmentally-friendly and therefore, offers an opportunity for rational use of insecticides in malaria control (Warui and Mutinga, 1994).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Magnitude of the malaria problem in Africa

Malaria remains one of the most devastating diseases occurring in the world today. Every year, an estimated 300-500 million new infections and 1-3 million deaths result from malaria (Breman, 2001; Greenwood and Mutabingwa, 2002; Snow *et al.*, 2005; Muturi *et al.*, 2008). More than 90% of these cases occur in sub-Saharan Africa mostly among the poor without access to health facilities (Muturi *et al.*, 2008). In some parts of Africa, people receive 200 to 300 infective bites per year, which is considered a very high rate of vector: man contact (Struchler, 1989; WHO, 1992; Bier *et al.*, 1994; Smith *et al.*, 2007). Malaria accounts for 20% to 50% of all admissions in all African health facilities and severely affects pregnant women, young children and migratory populations because of their low or non-existent immunity to the disease (WHO, 1996). About 1 million deaths among children in sub-Saharan deaths are attributed to malaria (WHO, 1996; Enami, 2003; WHO, 2003; Greenwood *et al.*, 2005). In Kenya, malaria is one of the major public health problems and about 70% of the population is exposed to the deadly *P. falciparum* (MOH, 2010). The disease is hyper-endemic and accounts for nearly 13.4% of all deaths and 35% outpatient cases (MOH, 2010). The frequent occurrence of malaria epidemics in the highlands east west of Rift Valley with a population of over 8 million further adds to the malaria risk in the country (MOH, 2001; MOH, 2010).

Despite an increasing effort to reduce the transmission and enhance treatment, there has been little change in malaria transmission in endemic areas since 1992 (Hay *et al.*, 2004). It is estimated that, if the prevalence of malaria stays on its present upward course, the death rate could double in the next two decades (Sachs and Malaney, 2002). About 11% and 4.3% of school days per year is missed by primary and secondary school pupils respectively due to malaria morbidity (Leighton and Foster, 1993). An estimated 170M working hours are lost each year due to malaria while 4 – 10 million school days may be lost due to the disease (MOH, 2010). Malaria places a heavy burden on the country's economy in general. The cost of drugs and patient care during admission are direct health expenditure (MOH, 2010). Besides, individual expenditure can not be quantified by the hundreds of thousands of cases that do not reach health facilities and resort to home management through purchase of drugs over the counter, cost of transportation to health facilities and worse of all, funeral expenses (MOH, 2001).

In an effort to address the rising malaria menace, the Government of Kenya through the Ministry of Health has developed a 10 year National Malaria Control Strategy (MOH, 2001) within whose framework the global Roll Back Malaria strategy is housed (RBM, 2000). The institutional framework to co-ordinate stakeholders' effort, strengthening of partnership and focus on national commitment in malaria control are emphasized in that strategy (MOH, 2001). As such, the development of new products like the natural pyrethrum-formulation for use in ITN is a positive development towards reducing the malaria burden in the country. The study thus presented a strong case for the need to explore preventive measures especially those targeting the vector mosquitoes through use of ITNs technology.

2.2 Distribution of malaria vectors in Africa

Understanding the vector species distribution is critical to any vector control programme that seeks to be efficient and successful. For effective ITN's, such vectors need to have preference for indoor resting and feeding habit (Petrarca *et al.*, 1991; Githeko *et al.*, 1996b). In Africa, malaria transmission is mainly by the *An. gambiae* complex and *An. funestus* Giles. *An. gambiae* complex consists of six sibling species, namely *An. gambiae sensu stricto* Giles *(An. gambiae* s.s.), *An. arabiensis* Patton, *An. merus* Donitz found in the Eastern Coast of Africa, and the *An. melas* found on the western coast of Africa. The fifth sibling species of this complex is *An. quadrimaculatus* Theobald found in Southern Africa and the sixth being *An. bwambae* found in Uganda (Davidson *et al.*, 1967; Gillies and DeMeillon, 1968).

In Kenya, *An. gambiae* s.s. mainly occupies the Nandi foothills but extends up to the shores of Lake Victoria (Service, 1978; Highton *et al.*, 1979). The species has also been identified in the coastal region, but absent from rice growing lowland plains of Ahero, Mwea and Taveta (Service, 1978; Highton *et al.*, 1979; Githeko *et al.*, 1996b). Like any parasitic disease with a complex cycle, distribution of malaria depends heavily on natural environment, physico-geographical and bio-geographical factors, determinants whose importance in transmission has long been recognized (Ross, 1911). The African region consists mainly of areas where transmission is intense and stable or of areas with irregular, unstable transmission with epidemic episodes. In the sub-Saharan Africa, *An. gambiae* s.s. and *An. arabiensis* have been recognized as the most important members of the *An. gambiae* complex, associated with most of the malaria transmissions (Gillies and Coetzee, 1987; Hunt *et al.*, 1998). These species have been widely distributed and can occur together over extensive areas. They share

fairly similar ecology, breeding in shallow, open, sunlit freshwater pools (Gillies and Coetzee, 1987; Hunt *et al.,* 1998). In Kenya, the two species have been reported to occur in sympatry in some areas though with proportional differences (Magak, 2004). *An. funestus* Giles is an important malaria vector, in some areas, playing a more important role than *An. gambiae* Giles and *An. arabiensis* Patton (Mbogo *et al.,* 1995; Githeko *et al.,* 1996b; Fontenille and Lochoven, 1999). The species is highly anthropophilic (Gillies and DeMeillon, 1968) and this has been found breeding in the western Kenya lowland and highland areas (White *et al.,* 1972; Ijumba *et al.,* 1990). To date, there is no documented evidence on the response of these key vectors to the natural pyrethrum formulation developed for ITN, a factor that is important for its successful usage in vector control.

2.3 Insecticide-Treated Nets (ITNs)

Mosquito nets and their impregnation with insecticides to improve effectiveness has a long history (Gillies and DeMeillon, 1968; Brun and Sales, 1976; Lindsay and Gibson, 1988; Sexton *et al.,* 1990.; terKuile *et al.,* 2003). It was recognized that untreated bednets were not fully effective in reducing incidents of malaria because mosquitoes often entered the nets when they were incorrectly erected or were torn (Darriet *et al.,* 1984; Enami, 2003). Research has demonstrated that treatment of bednets with insecticide makes the net more effective in preventing or deterring mosquito bites (Bogh *et al.,* 1998; Gimnig *et al.,* 2003; Kaburi *et al.,* 2009). ITN's can kill adult mosquitoes directly or force them to undertake longer, more harzadous foraging in search of veterbrate and aquatic habits (Killeen and Smith, 2007). Also the excito-repellent properties of some insecticides used in ITN's can reduce the frequency with which mosquitoes successfully acquire human blood (Taylor *et al.,*

1981; Roberts *et al.*, 1997) hence resorting to other animals that do not host the malaria parasite resulting in greatly reduced prevalence of sporozoite infection (Killeen *et al.*, 2007). Vector control through large-scale use of ITN's is a major component in the overall strategy of malaria control especially in Africa (Lines, 1996; Lengeler, 2004). Their use was reported to have reduced malaria incidence in children by upto 63% in Gambia (Alonso *et al.*, 1991). In Tanzania, (Shellenberg *et al.*, 2001) observed that consistent use of ITN's reduced parasitaemia in young children by 62% and increased child survival by 27%. Locally in Kenya, studies have shown the effectiveness of ITNs in reducing malaria infections (Sexton *et al.*, 1990.; Beach *et al.*, 1993; terKuile *et al.*, 2003). ITNs have therefore been recognised as a major intervention in vector control by the Roll Back Malaria initiative (Carnevale *et al.*, 1988). African Heads of States in the Abuja Declaration of 2000, stated that by 2010 60% of pregnant women and children under five should be sleeping under ITNs (RBM, 2000). Currently, the WHO-pesticides evaluation scheme (WHOPES) recommends pyrethroids as the insecticides for use on nets because of their fast knockdown action, excito-repellency and relative safety (Zaim *et al.*, 2000). The main active ingredients being, Alpha-cypermethrin (SC 6%), Cyfluthrin (EW 5%), Deltamethrin (25%, SC 1%) Etofenprox (EW 10%), Lamdacyhalothrin (CS 2.5%), Permethrin (EC 10%) (WHO, 1997; WHO, 1998; Zaim *et al.*, 2000). The *Anopheles* vectors in this region fit as targets to this technology because of their anthropophilic and endophagic behaviour (Githeko *et al.*, 1996b). However, one major challenge to the continued successful use of ITN is that resistance to pyrethroids already exists in several vectors of malaria in many countries including Burkina Faso, Cote d' Ivoire (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999; Diabate *et al.*, 2004), Ghana (Adasi *et al.*, 2000), Kenya (Vulule *et al.*, 1999; Ranson *et al.*, 2000; Stump *et al.*,

2004), Malawi (Hunt *et al.*, 2010), 2010, South Afriaca (Hargreaves *et al.*, 2000) and Turkey (Kasap *et al.*, 2000). Thus, the search for alternative insecticides that are effective and safe becomes a priority. Based on its outstanding properties and its long history of use against a wide range of insects (Tarimo, 1974; Bry *et al.*, 1977) and use on wall cloth and other fabrics (Bry *et al.*, 1977; Warui and Mutinga, 1994) pyrethrum when in a suitable formulation offers a potential to fight resistance and improve malaria control through ITNs.

2.3.1 Dose-response relationships study in ITNs

Laboratory baseline data on dose–response relationship between an insecticide treatment and target organism fulfills an essential first step in formulating field treatment requirements (Frommer *et al.*, 1983). Knockdown and mortality rates of an insecticide gives numerical measure of its effectiveness. This can be used in structure-activity correlation, resistance measurements and general decision making including optimizing the cost–benefit ratio of a product or determination of a more acceptable and efficient formulation (Moyses and Gfeller, 2001). In particular, a threshold is determined after which increments in dose of the insecticide administered elicit little and eventually no increase in response at all. While dose-response dependent effects have been studied in permethrin-treated nets (Beach *et al.*, 1993; Corbel *et al.*, 2004) no such studies on natural pyrethrum formulation-impregnated on nets have been documented. The need to determine dose-response model in relation to time of knockdown and kill of mosquitoes after exposures on to natural pyrethrum formulation treated nets was, therefore, necessary. This enabled generation of dose–response models/trends for determination of median lethal and optimal net treatment doses with the natural pyrethrum formulation.

2.3.2 Effect of different fabrics and colours of netting materials on bio-efficacy of ITNs

There are various netting fabrics and insecticide formulations that are used in insecticide-treated nets (ITNs). The most common fabrics are polyester, nylon and cotton (WHO, 2006; Murari *et al.*, 2007). For ITNs, the WHO pesticides evaluation scheme recommends only pyrethroid insecticides in various formulations such as emulsifiable concentrates (EC), suspension concentrates (SCs), emulsion oil in water (EW) and microcapsules (Zaim *et al.*, 2000). While studies elsewhere, have shown that differences in bio-efficacy of insecticide-treated nets may closely be linked to the nature of fabrics, among other properties (WHO, 1986; Rozendaal, 1989; Curtis *et al.*, 1996; Rafinejad *et al.*, 2008) in results of various studies point out to the possible link between specific insecticide formulation and the empirical bio-efficacy (Lines *et al.*, 1987; Hossain *et al.*, 1989; Vatandoost *et al.*, 2006). In another study, the efficacies of permethrin-treated nylon and cotton nets were compared and it was observed that 2.5 g/m^2 on nylon and 5.0 g/m^2 on cotton was required to prevent mosquitoes from biting human arm (Hossain *et al.*, 1989). It was further demonstrated that cotton net was not as effective as nylon nets treated with permethrin against *An. gambiae* and *An. funestus* (Lines *et al.*, 1987). However, in a separate study, it was demonstrated that polyester net gave maximum mortality over nylon and cotton nets when treated with lambda-cyhalothrin against *An. stephensi* (Vatandoost *et al.*, 2006) suggesting differential response of netting fabrics to different insecticides. In general, cotton absorbs more emulsion than nylon and polyester nets, which are light and flexible and, therefore, take less insecticide emulsion (Rafinejad *et al.*, 2008). The net texture has also been observed to influence the choice of nets by users (Murari *et al.*, 2007) although with little consideration to its influence on bio-efficacy of the nets.

Therefore, to improve on the use of ITNs in malaria control, a better understanding of the interaction between intrinsic fabric properties, formulation, impregnation dosage and empirical bio-efficacy of nets is necessary (MacCormack and Snow, 1986; Rafinejad *et al.*, 2008). Besides, the nature of the make-up of their woven net material, mosquito nets also come in different colours including white, blue and green. Millions of coloured nets are currently manufactured and will have to be treated by conventional dipping (Duchon *et al.*, 2006). In addition, coloured nets are purchased more by institutional buyers than white nets (Duchon *et al.*, 2006). While the choice of colour of a net may be linked to certain socio-cultural practices in some countries or communities (MacCormack and Snow, 1986; Itoh and Kurihara, 1992), the possible influence of the colour of a net on biological efficacy of insecticide-treated nets remains elusive. Recent work, although not conclusive, reported differential efficacy in nets in relation to colour, and that a single wash on coloured nets reduced the mortality to below 80% in deltamethrin-treated nets as opposed to white nets (Duchon *et al.*, 2006).

In other studies, the role of colour of a substrate in influencing the choice of a resting surface for an insect has been observed in tsetse flies (Dean *et al.*, 1968). This behavioral response has been used to develop fly traps and targets for control and population studies in tse-tse flies (Odulaja *et al.*, 1998).

To date, information on the influence of the type of netting fabric and colour on bio-efficacy of natural pyrethrum formulation-treated bednets is lacking. In addition, evaluation of the effectiveness of this formulation against different vector mosquitoes would be crucial in its usage in malaria control. Pyrethrins obtained from pyrethrum plant, *Chrysanthemum cinerariaefolium*, are a group of six esters formed by a

combination of two acids i.e. chrysanthemic and pyrethric acids and three keto alcohols i.e. pyrethrolone, cinerolone and jasmolone (Casida and Quistard, 1995). The structure of the active ingredient gives pyrethrins unique properties for use in different formulations for vector control.

2.3.3 Persistence and effect of washing on efficacy of ITNs

Studies on residual persitence and wash-fastness of a treated net is crucial in determining the appropriate retreatment period. To be fully effective, bednets need to be re-treated with insecticicde once or twice a year (www.who.int/tdr/research/final reps no4 .htm 1998). Washing of a net directly impacts on the dosage of contactable insecticide remaining on a net. It eventually reduces the insecticide dose to a level with minimal entomological impact (Lindsay *et al.*, 1991; Ordonez-Gonzalez *et al.*, 2002). Earlier studies have also shown that insecticide's active ingredients responded differently to washing. For instance, it was observed that deltamethrin and permethrin were more wash resistant than etofenprox and bifenthrin (Rafinejad *et al.*, 2008). There is however, scanty information on residual efficacy and wash resistance of nets treated with natural pyrethrum formulation and this was addressed in the current study.

2.3.4 Mosquito vector resistance to pyrethroid insecticides used in ITNs

Insect resistance is a change in an insect population overtime that results in their ability to withstand dosages of a given insecticide that were previously effective in killing them (Cochran, 1995). In malaria vectors, it has become a major concern for public health authorities and national malaria control programmes in Africa that rely on insecticides for vector control (WHO, 2003). Pyrethroids resistance in *Anopheles* vectors has been widely reported in many countries as previously indicated. Several

mechanisms have been previously implicated to be contributing towards resistance to pyrethroids by these vectors. However, it has been generally accepted that the underlying insecticide resistance mechanism include target site mutations in structural genes of the central nervous system such as gamma-amunobutyric acid (GABA) receptors, acetylcholinesterase, sodium channels and increased metabolic detoxification of the insecticide through elevated carboxylestarases, cytochrome P450 oxidases or glutathione transferases enzymatic activities (Hemingway *et al.*, 2004). Pyrethroids act on the nervous system by modifying gating kinetics of voltage sensitive sodium channels (Lund and Narahashi, 1983). *Anopheles* spp. resistance to pyrethroids is mainly associated with target site insensitivity arising from single point mutation in the sodium channel gene, often referred to as knockdown resistance (*kdr*) characterized by a leucine-phenylalanine mutation in West Africa ((Martinez-Torres *et al.*, 1998) and leucine-serine mutation in East Africa (Ranson *et al.*, 2000).

Pyrethroids and pyrethrins are structurally related molecules and further reports have linked *kdr*-type to protect certain insect vectors from pyrethrum (Scott and Matsumura, 1981; Scott and Matsumura, 1983.). However, one way of fighting resistance is to incorporate synergists in an insecticide formulation. Synergists may act through competitive inhibition of a family of metabolic enzymes called P450s or altering cell membrane or hindering site accessibility to the compound (PBK, 1987). The role of a synergist and an anti-oxidant on *kdr* resistance is not known. There was need therefore to evaluate the effect of the synergized natural pyrethrum formulation on *kdr* resistant mosquitoes and to monitor the spread of *kdr* mutation within given mosquito population as part of the overall vector control strategy.

2.3.5 Influence of ITNs on mosquito behaviour and other entomological indices

Mosquito behaviour cycles between host-seeking, feeding, resting, oviposition site-seeking and back to host (Saul *et al.*, 1990). Successful feeding is one of the three possible outcomes of a host encounter by a female vector. The other two alternatives for a mosquito is death, while attempting to feed and diversion, to seek another host (Killeen *et al.*, 2007). Diversion includes the combined effects of non-contact repellency, and contact mediated repellency often referred to as excito-repellency (Roberts *et al.*, 2000). The excito-repellent effect of an insecticide could lead to reduced human biting habit, biting rate and vector longevity thus affecting the vectorial capacity of a mosquito (Garret-Johnes and Shidrawi, 1969; Taylor *et al.*, 1981). The irritant effect of certain insecticides can reduce man-mosquito contact in houses and divert mosquitoes to feed on domestic animals sheltered near dwellings of human (Killeen *et al.*, 2007).

Work under laboratory conditions have shown the effectiveness of nets treated with natural pyrethrum formulation (Sum *et al.*, 2011), however, these evaluations were mainly conducted under laboratory conditions on one sibling species, *An. gambiae* s.s. While the sibling species are indistinguishable morphologically, they differ in certain behavioural and ecological attributes that are important in their vectorial capacity for malaria (Gillies and Coetzee, 1987; Githeko *et al.*, 1996a) thus calling for multiple evaluations on their response to specific insecticide formulation. Elsewhere studies have demonstrated differential responses of different vector mosquitoes to insecticides. For example, a previous study (Mosha *et al.*, 2008) reported that nets treated with permethrin offered highest personal protection against *An. arabiensis* (61.6% reduction in fed mosquitoes) while Deltamethrin and alpha-Cypermethrin

provided lower personal protection (46.4% and 45.6 respectively) against the same species. In an earlier work (Jawara *et al.*, 1998), it was observed that *An. gambiae* s.l. were killed by d-cypermethrin than by permethrin or *lamda*-cyhalothrin. The differences may be attributed to specific feeding and resting behavioural patterns of the vector mosquitoes which may greatly influence their reaction to the specific active ingredient in the ITN (Mosha *et al.*, 2008). In Kenya, the key malaria vector *An. gambiae* s.l. is found in large parts of Western Kenya (Collins *et al.*, 1988; Mukiama and Mwangi, 1990 ; Githeko *et al.*, 1992) and has been known to be almost exclusively endophagic (Gillies and DeMeillon, 1968) while *An. funestus* is also widely found in many parts of Kenya) and is highly anthropophilic (Gillies and DeMeillon, 1968) *An.arabiensis* which occurs mainly in the plains (Mukiama and Mwangi, 1990; Githeko *et al.*, 1992) is a vector known for its partial zoophilic and exophilic behaviour (Mosha *et al.*, 2008). The emergence of new vector behavioural phenotypes is a less recognized phenomenon than insecticide resistance though it has the potential to similarly diminish the effectiveness of current interventions (Ferguson *et al.*, 2010).

Despite the demonstrated potential for use of natural pyrethrum in ITNs, to date there has not been an evaluation of the performance of the nets treated with the natural pyrethrum formulation against wild common vector species under field situation. In addition, there is lack of information on the behavioural responses in terms of feeding and resting indoors of these common vectors in houses where there is natural pyrethrum-ITNs.

2.4. Pyrethrum plant and its historical use in malaria control

Pyrethrum plant (*Chrysanthemum cinerariaefolium*) is a perennial plant of the genus *Chrysanthemum* which produces daisy-like flowers (Plate 1). Its commercial cultivation as an insecticide in 1840 in Dalmatia (now Yugoslavia) is well documented (Moore and Levy, ; Casida and Quistard, 1995). However, Kenya has been the leader with a production that once went as high as 18000 MT Pyrethrum (PBK, unpublished reports). Pyrethrum is also grown in Tanzania, Rwanda , DRC, New Guinea and Tasmania (Glynne-Jones, 1973; Moore and Levy, 1975).

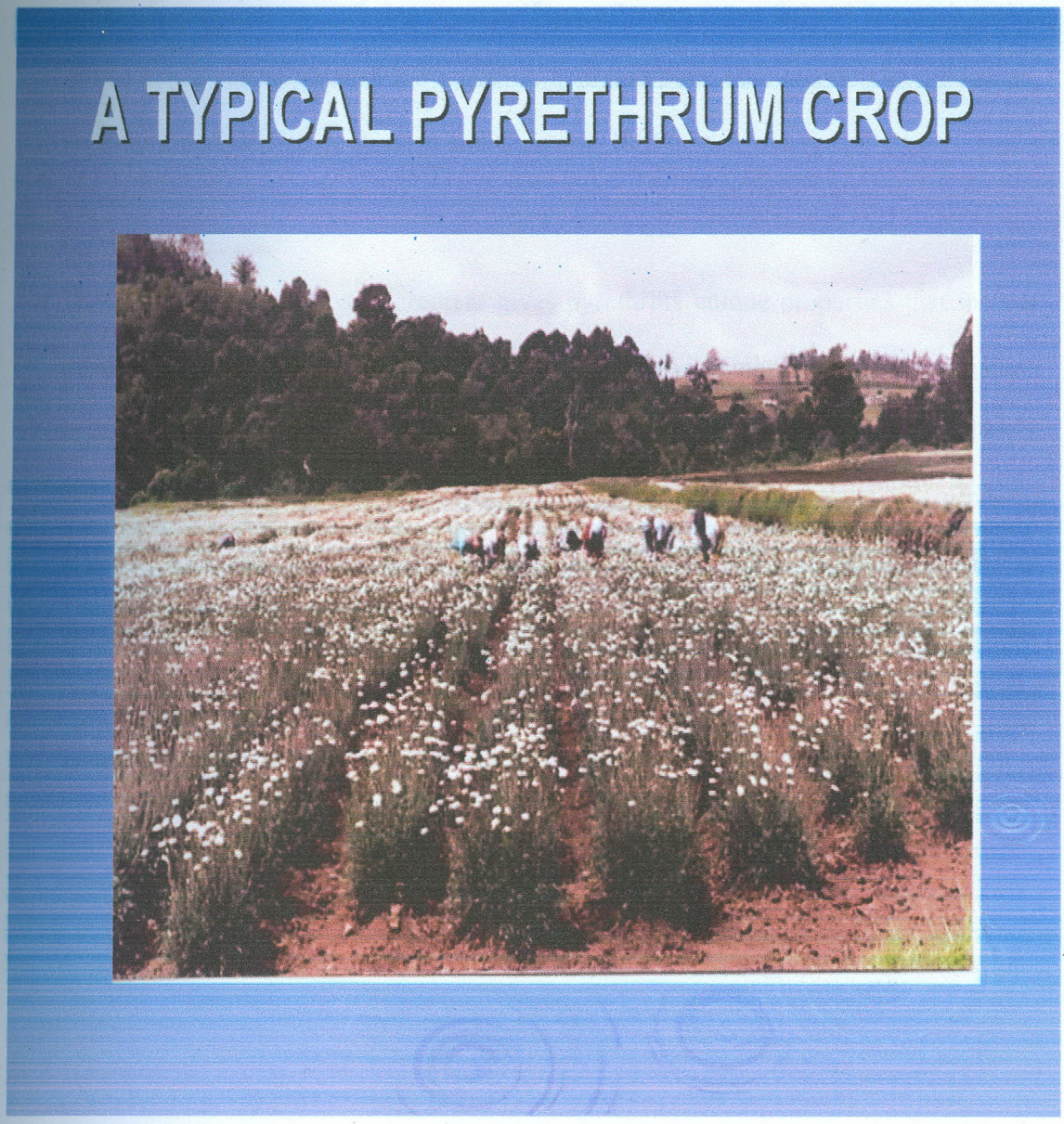

Plate 1: A typical pyrethrum field with farmers picking ready flowers

The active ingredients, pyrethrins, which is derived from its dry flowers through a series of extraction and refining process (Figure 1), are esters formed by combination of two acids; chrysanthemic and pyrethric acid and three keto-alcohols-pyrethrolone, cinerolone and jasmolone (Casida and Quistard, 1995).

The structure of the active ingredient gives pyrethrins unique properties that include rapid action on insects, non-persistence in an open environment, and low mammalian toxicity (Sattelle and Yamamoto, 1988; Metcalf, 1993; Casida and Quistard, 1995). The unique combination of the esters also makes pyrethrins molecule less prone to selective resistance by mosquitoes and other insects. This resistance ability of mosquitoes to different insecticides was also investigated in the current study. Despite its local availability and its unique properties, pyrethrins have not been used in emerging technologies like ITN, which is key to tackling the malaria menace.

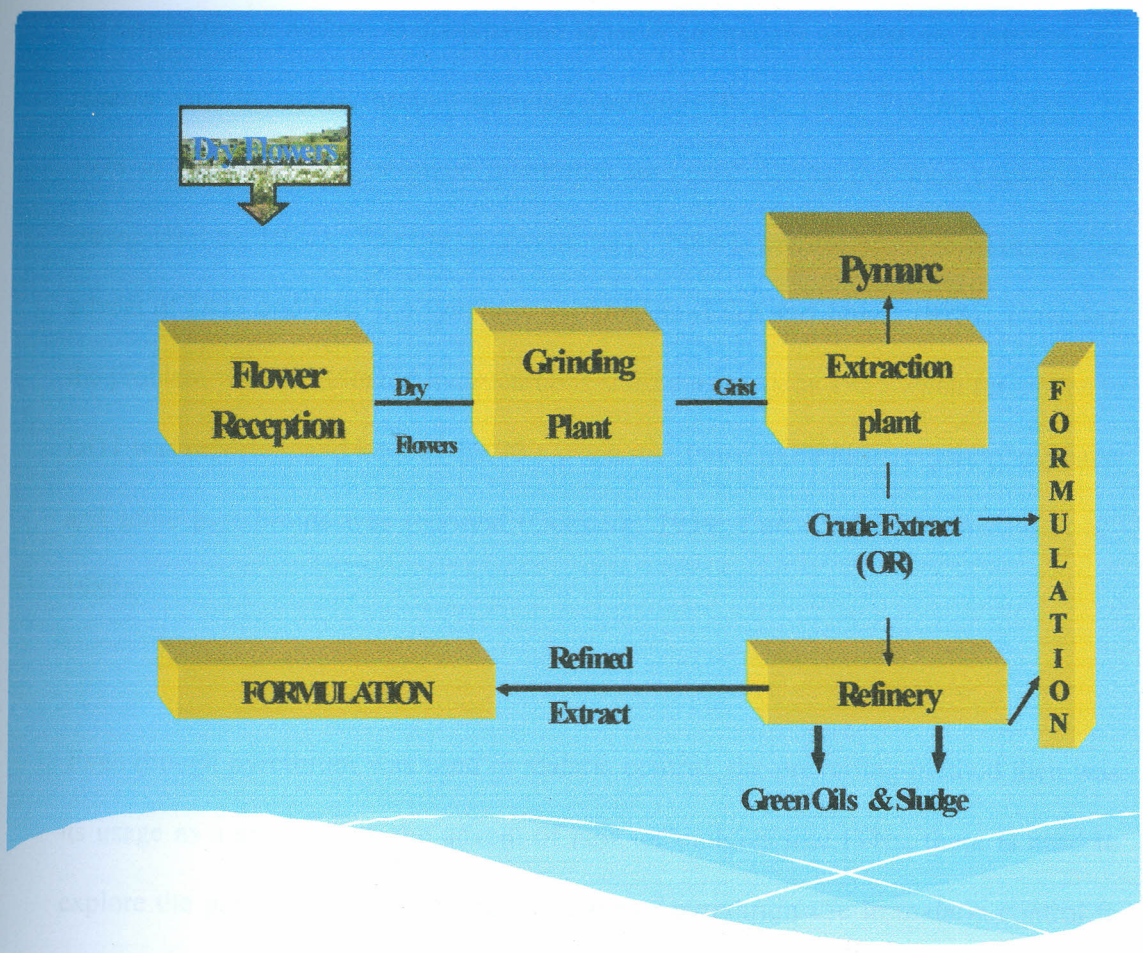

Figure 1: Stages in pyrethrum extraction and refining (Casida and Quistard, 1995).

Pyrethrum use in malaria is documented in early campaigns against *An. gambiae*, *An. funestus* and *An. melas*, through spraying dwellings (Ross, 1911; Thornton, 1935). In 1974 in El Salvador, synergized pyrethrum was used during the main transmission season (Hobbs, 1976). The use of pyrethrins in malaria control diminished during the advent of DDT and later the synthetic pyrethroids (Eliot and Janes, 1978). However, the problem with chlorinated hydrocarbons started as early as 1959 when resistance to DDT was reported, and later in the 70's when resistance to dieldrin, organophosphates and some pyrethroids was reported (Gokberg, 1959; Curtis, 1962; Ramsdale *et al.*, 1980).

Even though pyrethrum was used in malaria control, the prevailing method then was its usage as a spray. With the advent of new technolgies like ITNs, there is need to explore the possibilty of expanding the scope of pyrethrum use in malaria control to exploit its natural potential. The use in ITNs requires a stabilized and synergized natural pyrethrum formulation to enhance its variuos properties.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Determination of the effect of different net impregnation doses of the pyrethrum formulation on knockdown and kill of mosquitoes and optimal dose for field application.

3.1.1 Test mosquitoes

Laboratory bioassay tests were carried out on 3 day-old female *An. gambiae* s.s. Kisumu strain, reared at Kenya Medical Research Institute (KEMRI) - Centre for Global Health Research (CGHR) laboratory. The mosquitoes were bred in an insectary kept at room temperature (26-27°C) and relative humidity of 75-80%. The larvae of the mosquitoes were fed on grain and yeast tablets purchased locally. Adults emerged after about 10 days and females were sorted out for tests while others were fed on rabbit blood for egg laying purposes in specialized cages to continue the generation.

3.1.2 Formulation of the pyrethrum-emulsifiable concentrate

An emulsifiable concentrate (EC) natural pyrethrum formulation containing 5% pyrethrins, weight per volume (w/v) as the active ingredient (a.i), mixed with a synergist (25%), food grade anti-oxidant and a non-ionic emulsifier was formulated at the Pyrethrum Board of Kenya (PBK) Entomology laboratory. The active ingredient was obtained from a 25% pyrethrins (w/v) refined extract manufactured at the PBK factory while the synergist, emulsifier, and anti-oxidant were commercial grade obtained from manufacturers (Endura Spa, Italy and Bayer AG, Germany). The active ingredient content in the formulation was determined to be 5±0.5% through high performance liquid chromatography (HPLC) using a Varian Vista series 5000

liquid chromatograph. In the analysis, 0.1g of the sample was put in 100ml volumetric flask and hexane solvent added to the mark. The mixture was thoroughly shaken to achieve homogeneity, then 10 microlitres was injected into the system manually at the set flow rates of 8ml/minute, peak detector at 230nm, column factory packed CN10, run-time 10 minute and chart speed at 0.25cm/minute. The % pyrethrins were then determined based on the shape and retention time of the peaks on the chromatogram, and quantified against the curves of analytical world standard pyrethrum extract (WSPE).

3.1.3. Impregnation of the test mosquito net

A commonly used fabric in ITN, multi-filament polyester netting of 100 deniers strength of 20 holes/cm^2 manufactured by Siam Dutch mosquito netting company, Thailand, were purchased from local stores in Kisumu City and used in this study. The nets were impregnated with the pyrethrum formulation to deposit respective target doses of pyrethrins equivalent to 75 mg/m^2, 100 mg/m^2, 250 mg/m^2, 500 mg/m^2 and 1000 mg/m^2, based on standard impregnation procedures (Miller *et al.*, 1999). Briefly, the pieces of nets measuring 2 m^2 were cut and, before impregnation, their liquid absorbency was first determined by weight after normal wringing. Before impregnation, the amount of stock solution (X) of pyrethrins 5% emulsifiable concentrate (EC) needed to deposit a target dosage was determined as follows:

$$\frac{x = Target\ dosage\left(\frac{mg}{m2}\right) x\ Area\ of\ the\ net\ (m2)}{Conc.\ of\ stock\ insecticide\ \frac{g}{kg}}$$

The nets were then carefully soaked in a non-absorbent plastic bucket containing the mixed concentrate in a 'dip-it-yourself' manner (Plate 2). The fingers were protected with plastic gloves and impregnation properly executed in order to ensure uniform distribution of the solution onto the net. The nets were then left to dry for one-day in a dark room.

Plate 2: A piece of white netting material being impregnated with pyrethrum formulation

The quality of impregnation was assessed by taking two pieces of nets measuring 10 cm x10 cm from the treated nets and presented for instrumental chemical analysis at the PBK chemical laboratory using a high performance liquid chromatography (HPLC) machine. Before analysis, the pieces of net were weighed, soaked in 40 ml of hexane in a beaker. The extract was shaken for 10 minutes then transferred into a volumetric flask connected to a condensor where the sample was subjected to a hot extraction of pyrethrins for 8 hours. After extraction, 50 microlitres was injected into the HPLC machine for determination of the concentration of pyrethrins as described in 3.1.3. All netting pieces analysed achieved a minimum of 95% of the target dose and thus ascertained the integrity of the impregnation process.

3.1.4 Evaluation of the efficacy of nets treated with natural pyrethrum formulation on knockdown and kill of *An. gambiae* s.s.

Bioassays were conducted in accordance with the Word Health Organization guidelines (WHO, 1988). In the tests, pieces of nets measuring about 15 cm^2 treated with the various doses of pyrethrins were cut and strapped onto one end of transparent plastic WHO cones using clips. Twenty five (25) female *Anopheles* mosquitoes aged 3 days old were introduced into the cups and held in horizontal position so that the treated pieces of nets are in vertical position simulating the actual bednet situation. . The mosquitoes were then exposed for 3 minutes and subsequently transferred to holding cups. Level of Knockdown (KD) was monitored every 3, 5, 10, 15, 30 and 60 minutes. Thereafter, the mosquitoes were put in plastic jars, provided with sucrose pads and held in the recovery room for mortality assessment after 24 hours. Four replicate tests were conducted on each dose simultaneously under room temperature. Mosquitoes exposed to untreated nets as well as bednets treated with permethrin 20%

emulsifiable concentrate (PERIPEL™ 20 EC, Agrevo, formerly Russel at the recommended dose of 500mg a.i/m^2 was used as negative and positive controls, respectively.

3.1.5 Determination of dose-response relationship of nets treated with natural pyrethrum formulation against *An. gambiae* s.s.

The response of the mosquitoes in terms of knockdown and kill in relation to the different doses of natural pyrethrum-treated nets was determined from the bioassays. A logit (i.e. log-logistic) regression analysis was used to study the insecticide dose-response relationship.

3.2 Assessment of the effect of netting fabrics and colour on bio-efficacy of nets treated with natural pyrethrum formulation against *An. gambiae* s.s.

3.2.1 Netting materials and impregnation

Three multi-filament rolls of netting fibres of nylon, polyester and cotton of 100 deniers strength with mesh size of 25 holes/cm^2 (Siam Dutch mosquito netting company, Thailand), were purchased from local stores in Kisumu City and used in this study. The impregnation process was as earlier described (Miller *et al.*, 1999). Briefly, pieces of nets measuring 2 m^2 were cut and, before impregnation, their liquid absorbency was first determined to be 70 ml/m^2 for nylon, 75 ml/m^2 for polyester and 225 ml/m^2 for cotton by dipping a sample in water. The nets were then impregnated with pyrethrins as described in section 3.1.3 and the respective deposit doses equivalent to 100 mg/m^2, 250 mg/m^2, 500 mg/m^2 and 1000 mg/m^2, using the following amounts of stock natural pyrethrum-formulation i.e. 4 ml, 10 ml, 20 ml and 40 ml, respectively.

3.2.2 Efficacy of the natural pyrethrum formulation impregnated on the three netting fabrics on knockdown and kill of *An. gambiae* s.s.

After impregnation, the three different treated pieces of fabrics, nylon, cotton and polyester, measuring 15 cm^2 were cut and strapped onto one end of transparent plastic WHO cones using paper clips and bioassays conducted as described in 3.1.4. Four replicate tests were conducted concurrently per treatment and per fabric. Mosquitoes exposed to untreated nets or permethrin-treated nets at the recommended dosage were used as negative and positive controls, respectively. The bioassay tests were conducted within one week after impregnation.

3.2.3 Assessment of the effect of colour of pyrethrum formulation-treated nets on contact bio-efficacy against *An. gambiae* s.s.

For these studies, one of the most commonly used netting fabrics, polyester, of 100 deniers and mesh size of 25 holes/cm^2, was purchased from local stores. Three common colours of netting fabrics viz, white, blue and green were chosen for experimentation. Before impregnation, the nets were cut into pieces measuring 45 cm^2. Nets were and then impregnated following the procedure earlier described in section 3.1.3 in order to deposit pre-determined effective doses equivalent to 250 mg/m^2 and 500 mg/m^2. Knockdown and mortality effect resulting from tarsal or body part contact with the treated netting material was evaluated through bioassay as described in 3.1.4.

3.3 Determination of the effect of repeated washing and six months persistence of netting treated with natural pyrethrum formulation in relation to bio-efficacy against *An. gambiae* s.s.

3.3.1 Washing procedure and susceptibility bio-assays

A multi-filament polyester netting fabric of 100 deniers strength was conventionally treated as described in 3.1.3 with pyrethrins at the recommended dose of 500 mg/m^2. The net was cut into several pieces measuring 22.5cm x 22.5 cm before the same were subjected to 1-5 washes using local bar soap (white star® bar soap) with soft water. In the washing process, 2 g of the soap was dissolved in 500ml of rain water using magnetic stirrer for 30 min at 30°C. The dissolved solution was then poured into a small plastic basin and in a typical domestic washing style, the nets were rubbed ten times and rinsed twice with 500ml of water. After every washing, the nets were dried at room temperature in a shaded place and stored in aluminum foil before tests. Bio-efficacy evaluation to determine the retention of efficacy post-washing was based on standard procedure outlined in 3.1.4, after every wash-dry cycle up to a total of 5 washes. A total of 10 pieces of nets were washed, and of these, five were used in chemical analysis using WH Varian Vista Series 5000 liquid chromatograph 2005 as described above while the other five were used in bioassays using WHO procedures (WHO, 1998). The number of washes sustaining mortality and/or KD above cut-off point of 80% mortality after 24hrs and 95% KD after 60 minutes post-exposure to the treated net was then determined.

3.3.2 Efficacy, persistence and bio-availability of pyrethrins on treated nets

Residual efficacy persistence of pyrethrins on treated nets was monitored through 3-minutes bioassays performed on a monthly basis on the pyrethrins-treated nets at 250 mg/m^2 and 500 mg/m^2 for 6 months as described in 3.1.4.

3.3.3 Chemical persistence of pyrethrins on treated nets

Further evaluations were carried out monthly by chemical analysis, in which case, two pieces of treated nets, measuring 10 cm x 10 cm, were subjected to HPLC as described in 3.1.3.

3.4 Determination of the susceptibility and distribution of *kdr* resistance genes in wild *An. gambiae* s.l. in Kisian, Ahero and Kipsitet areas and pink eyed *An. gambiae* s.s. with *kdr* genes to the natural pyrethrum formulation

3.4.1 Study areas

The study was conducted in three areas, Kisian in Kisumu West District, Ahero in Nyando District, and Kipsitet in Kericho District, all being *P. falciparum* malaria holoendemic areas situated within Western Kenya (Stump *et al.*, 2004) as depicted in Appendix 1. The first two sites (Kisian and Ahero) separated by about 20 km, are located in the Lake Victoria region's climatic domain at an altitude of about 1170m above sea level (a.s.l.) and receive an annual rainfall of about 700mm. In addition, the main agricultural activity in Ahero area is irrigated rice cultivation and similar agricultural areas that have extensive insecticide use have been implicated in pyrethroid resistance in *An. gambiae* s.l. (Chouaibou *et al.*, 2008). Kipsitet, on the other hand, is located at the transition point of the lowland lake region warm climate of the Nyanza Province and the highland cool climate of the Rift Valley Province

situated at an altitude of 2000m a.s.l. The area receives high annual rainfall of up to 2000mm. This area therefore is prone to occurrence of epidemic malaria outbreaks and is usually among the target areas for IRS with pyrethroids in Kenya (MOH, 2001), thus may have mosquitoes exhibiting resistance traits. In all three study sites, houses have mostly mud walls and grass-thatched or iron sheet roofs typical of the Kenyan rural set-up.

3.4.2 Mosquito collection

Indoor-resting adult mosquitoes were collected based on procedures described by Service, (1980). Briefly, search was conducted in randomly-selected houses using spotlights and aspirators in the morning hours from 8.00am-11.00am in August 2010 as shown in Plate 3. The mosquitoes were initially identified morphologically as *An gambiae* s.l. or *An. funestus* as previously described (Gillies and DeMeillon, 1968). The mosquitoes were then put in paper cups and fed with 10% sucrose solution soaked in cotton. While in the laboratory, the mosquitoes were transferred into breeding cages in order to establish larger colonies from which the emerging adults were pooled together and samples used for molecular identification of the *An. gambiae* sibling species, genotyping for the presence of *kdr* genes and determining the susceptibility of wild mosquitoes species to the natural pyrethrum formulation and control insecticides.

Plate 3: Collection of indoor resting mosquitoes using a spotlight and aspirator.

3.4.3 Molecular identification and *kdr* genotyping

3.4.3.1 DNA extraction

The DNA extraction process was based on an earlier protocol ((Collins *et al.*, 1988). In the procedure, a +65 ^{oC} waterbath was prepared, then dry samples of 30 unfed adult mosquitoes from each study site were placed individually in sterile centrifuge tubes and crushed in 100µl of grinding buffers in a 4:1 ratio for homogenization buffer consisting of 0.25M EDTA, 2.5%$^{W}/_{V}$ SDS and lysis buffer consisting of 0.5M Tris Base, both mixed to a pH of 9.2, respectively. The ground samples were incubated in 65°C water bath for 30 minutes in order to denature nucleases that would further degrade DNA and provide optimum temperature for activity of lysis buffer. A volume of 14 µl of potassium acetate was then added and the samples vortexed to mix and then incubated in ice for 30 minutes and the supernatant transferred. The samples were then spun in an Eppendorf centrifuge-5415D for 10 minutes at 13,200 rpm and the supernatant removed and stored in sterile vials. A volume of 200µl of cold 90% absolute ethanol was then added and samples placed at -20°C for 20 minutes. A final spin was done for 20 minutes at 13,200rpm to pellet the DNA. The samples were then reconstituted in 100µl of TE buffer (0.001M EDTA, 0.01M Tris-HCl at pH 8.0) to remove any RNA that co-precipitated with DNA. The DNA pellets were then allowed to dry by inverting the tubes.

3.4.3.2 Molecular identification of *An. gambiae* species using polymerase chain reaction (PCR)

Conventional PCR following protocol modified from a previous study (Scott *et al.*, 1993) was used to distinguish between the two sibling species of the *An. gambiae* complex native. The protocol had the following ingredients: 5X GoTaq PCR buffer, Primers [for *An. gambiae* s.l. species identification , forward universal primer (5`-GCT GCG AGT TGT AGA GAT GCG-3`), reverse *An. gambiae* primer (5`-GCT TAC TGG TTT GGT CGG CAT GT-3`) were used, and for *An. arabiensis* a reverse primer (5`-GCT TAC TGG TTT GGT CGG CAT GT-3')], $MgCl_2$, dNTPs (deoxynucleoside triphosphates), Taq DNA polymerase per reaction as below (Table 1).

The reaction was ran on Perkin Elmer^{TM} GeneAmp PCR system 9600 for 30 cycles at 95°C for 30 seconds denaturation, 64°C at 30 seconds for annealing and 72°C for 45 seconds for elongation. The PCR products were resolved on a 3% agarose gel and visualized by Ultraviolet trans-illumination.

Table 1: The reaction mixture setup of the PCR master mix/sample

Reagent	Concentration for 1 sample	Volume per sample
PCR Water	n/a	5.525 µl
5X PCR buffer (No $MgCl_2$)	1X	3µl
2mM dNTP mix	0.2mM of each	1.5µl
25mM $MgCl_2$	1mM	0.6µl
Primer GA	1µM	0.6µl
Primer AR	1µM	0.6µl
Primer UN	1µM	0.6µl
Taq DNA Polymerase	0.075U	0.075µl
DNA template		1.5µl
Total		**14µl**

3.4.3.3 Genotyping for *kdr* genes

Real-time PCR (RT-PCR) was used to determine *kdr* genotype at the amino acid position 1014 of the voltage gated sodium channel following a method modified as described by Bass *et al.* (2007). In RT-PCR dNTPS, buffer, and Taq polymerase were all included in one commercial mix (2X concentration) together with allele-specific probes that bind to the PCR product during the course of the reaction and assist in distinguishing between the alleles. Samples were genotyped using probes for wild type (5'-CTTACGACTAAATTTC-3' labeled with HEX), and L1014S allele (5'-ACGCTGAATTTC-3' labeled with FAM) and L1014F allele (5'-ACGACAAAATTTC-3' labeled with FAM). RT-PCR reactions were run on a Stratagene MxPro 3000 machine using a 96-well format. Each reaction included 50 µl of 2X Taqman RT-PCR master mixes, 0.2µM *kdr* forward primer (5'-GCTGCGAGTTGTAGAGATGCG-3'), 0.2 µM reverse primer (5'-GCTTACTGGTTTGGTCGGATGT-3'), the wild type and L1014S probes at respective concentrations of 0.2 µM and 0.15 µM and 50ng DNA template. Each 96-well plate included positive controls for all three genotypes in triplicate along with non-template negative control. PCR conditions included initial melting step at 95°C for 25 seconds and annealing and elongation at 64 °C for 1 minute. Reaction curves (Figure 2) were visualized using the Stratagene MxPro QPCR software.

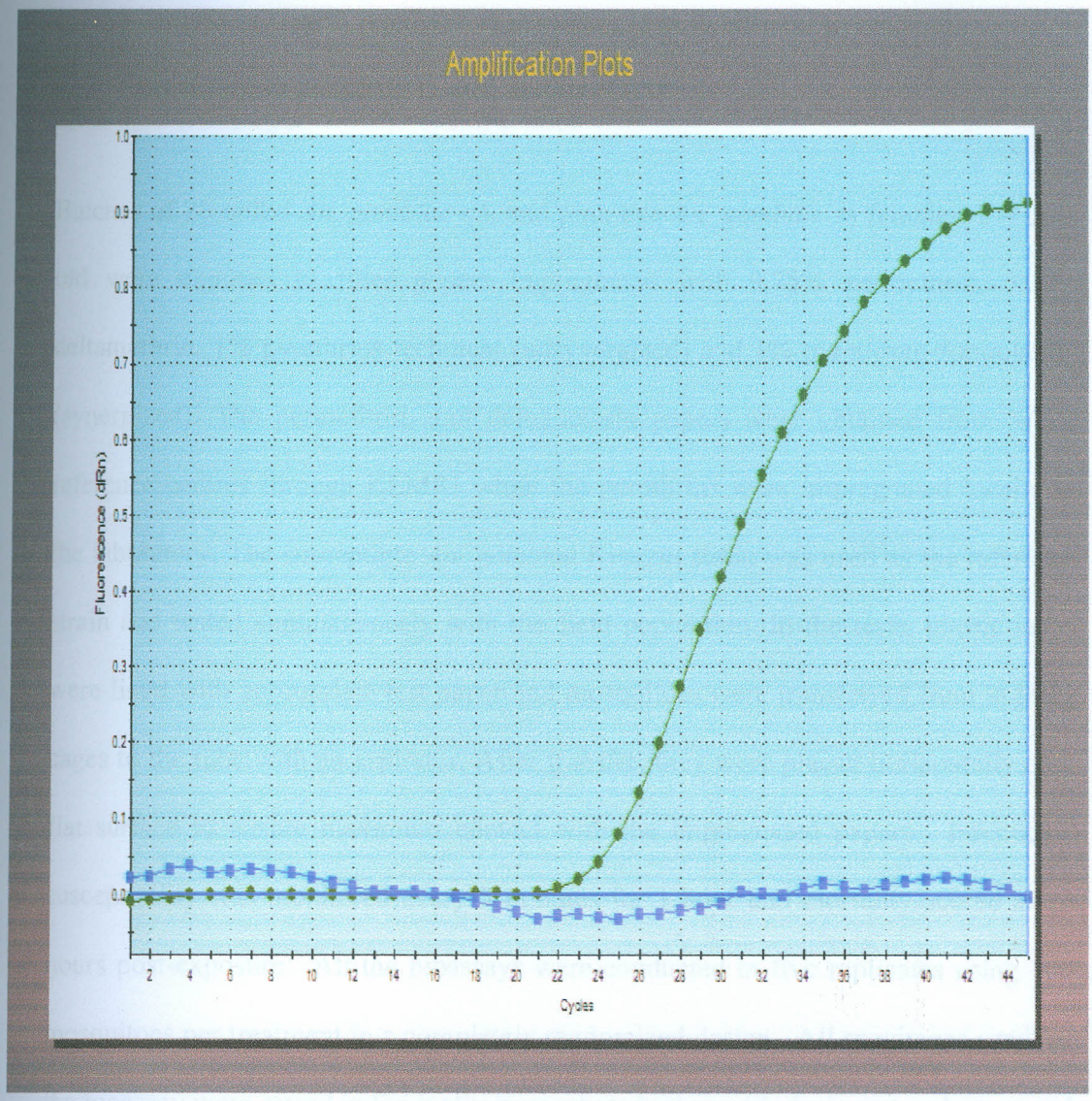

Figure 2: Susceptible sample plot from a RT-PCR analysis of *kdr* genes with different assay colours (Green for HEX and Blue for FAM).

3.4.4 Bioassays on susceptibility of the wild *An. gambiae* s.l. and pink eyed *An. gambiae* s.s. with fixed *kdr* resistance genes to natural pyrethrum formulation permethrin and deltamethrin

Batches of 20 unfed *An. gambiae* s.l. and pink eye *An. gambaie* ss females, 2-4 days old were exposed to filter papers impregnated with 0.75% permethrin, 0.05% deltamethrin, 1% pyrethrins technical (unsynergized) and 1% pyrethrum-formulation (synergized). The permethrin, and deltamethrin papers were obtained from WHO reference centres through KEMRI while the pyrethrins were impregnated locally in the laboratory. The susceptible *An. gambiae* Kisumu strain was used as the reference strain and tested simultaneously with the field population. In the tests, plastic tubes were lined with appropriate test paper and mosquitoes were transferred from holding cages to the tube with an aspirator. After transfer, they were placed horizontally on a flat surface to ensure maximum contact with the impregnated papers. Insecticide susceptibility was monitored every 5 minutes for 1 hour and mortality scored at 24 hours post-exposure. All the bioassays were conducted in five replicates using 100 mosquitoes per treatment in a completely randomized design. All specimens used for the bioassay were stored individually in numbered tubes with desiccant and preserved at 20°C freezer for further laboratory processing. Evaluation of the resistance/susceptibility status of the mosquito vectors followed criteria in which resistance was indicated by mortality rates below 80%, while mortality rates greater than 98% were indicative of susceptibility. Mortality rates of between 80-98% suggested increased tolerance (WHO, 1998).

3.5 Evaluation of the effect of bednets impregnated with natural pyrethrum formulation on mosquito behaviour (feeding, irritancy, and repellency), density and mortality under laboratory and small scale field situations

3.5.1 Laboratory evaluation of nets impregnated with natural pyrethrum formulation on feeding inhibition of mosquitoes using tunnel tests

Blood-feeding inhibition and irritancy of natural pyrethrum formulation impregnated nets to mosquitoes were studied in the laboratory based on a modified WHO procedure (WHO, 2006). The process involved releasing non-blood fed female mosquitoes, aged 3-5 days old in a tunnel (square section 25cm x 25cm) made of glass, and 60cm in length. At each end of the tunnel, a 25cm square cage was fitted (extension) and covered with polyester netting. At one third of the length, a disposable cardboard frame was placed with the netting sample measuring 45cm x 45cm treated at 250 and 500 mg/m^2. Each netting sample had nine holes of 1 cm diameter each with one hole located at the centre, while the other eight were equidistantly located 5 cm from the border. A total of 400cm^2 was available for possible contact with the mosquitoes. In the shorter section of the tunnel, static rabbit bait was placed and held in an immovable position (Plate4). In the cage at the end of the longer section of tunnel, 50 females mosquitoes in four replicates were introduced and left to fly freely in an attempt to make contact with or reach the bait through the holes. The tests were carried out at room temperature and under subdued light. The knockdown and immediate mortality was assessed by counting separately for each section of the tunnel after 24 hours. A total of 2 tunnels were used simultaneously with one tunnel being used with nets impregnated with pyrethrum formulation while the other tunnel was used with untreated netting (negative control). Blood-feeding

inhibition was assessed using the following formula; (NC-NT)/NC x 100 where NC and NT refers to number of blood-fed mosquitoes in untreated and treated tunnels, respectively.

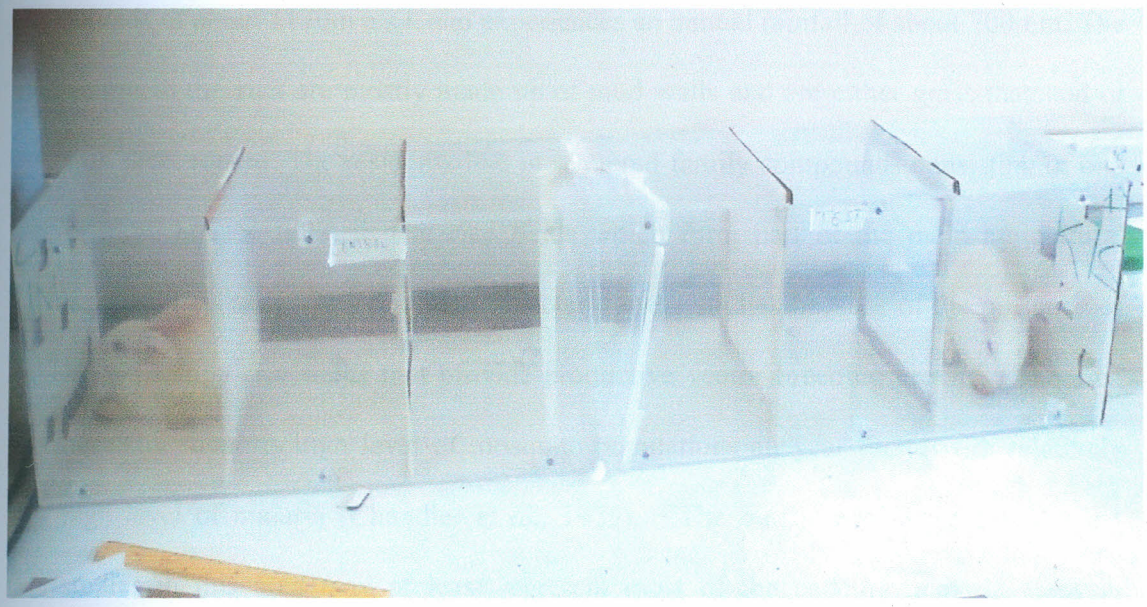

Plate 4: Modified WHO tunnel for feeding inhibition assay.

3.5.2 Evaluation of the effect of natural pyrethrum formulation impregnated nets on indoor resting density and feeding behaviour of *Anopheles* mosquitoes under field situation

3.5.2.1 Description of the study area for behaviour studies

The experiments were conducted in Kano area of Kisumu District about 15 km from Kisumu city. The area is located in the Lake Victoria region's climatic domain at an altitude of about 1170m a.s.l. and experiences an annual rainfall of about 700mm. The houses in the area are mostly made up of mud walls and are either grass-thatched or iron sheet roofed. The residents live in scattered family compounds consisting of one or more houses situated near rice fields which form part of the main agricultural activity. This area was principally selected for the studies because of the presence of large swampy rice fields that provide productive vector breeding grounds. The area normally supports high level of mosquito populations all year round with relatively high level of malaria (Chandler *et al.*, 1975). The outcome of the entomological studies in this area may at least represent most of the endemic malaria areas in Nyanza Province. In addition, the primary malaria vectors which are mainly *An. gambiae* s.l. and *An. funestus* are known to exist in the area (Gimnig *et al.*, 2003). Previously, entomological studies have been conducted in the area and information generated from such baseline studies can be used for purposes of comparison and to investigate the changes in vector habits and other behavioural patterns in response to the use of natural pyrethrum formulation-impregnated nets (Chandler *et al.*, 1975; Githeko *et al.*, 1996b).

3.5.2.2 Experimental Design

A total of 24 houses measuring about 10m x10m locally known as "simba" were randomly selected from the village based on the number of sleepers (1-2 people/bed/house), similarity in construction type and willingness of the owners to participate in the studies. The houses had eaves that allowed unimpeded entry/exit of mosquitoes. The selected houses were appropriately marked and coded for purposes of the field experiments. Twenty four (24) nets of polyester fibre of 100 deniers (Siam Dutch, Thailand) knitted in conical shape with dimensions of 250cm (height), 1050cm (circumference diameter) and 65cm (ring diameter) giving a surface area of 15.02 m^2 to fit ordinary medium size beds were locally purchased in Kisumu city stores. Eight (8) of the nets were impregnated with pyrethrum at the rate of 500 mg/m^2, another set of 8 with permethrin (PERIPEL^{TM} 20 EC, Agrevo, formerly Russel Uclaf) at the rate of 500 mg/m^2 to act as positive control while a last set of 8 nets were left untreated so that they act as the negative control in a completely randomized design. The nets were then distributed to the houses within the village separated with a distance of 5km to prevent inter-treatment movement of mosquitoes. All the nets were marked with water-soluble blue ink to detect any washing within the experimental period. Before distribution of the nets, the users were sensitized on proper use of the nets.

3.5.2.3 Impregnation of bed nets for field experimentation

Before impregnation, the liquid absorbency of an individual net was determined by weight after normal wringing to be 750 mls. Nets were then impregnated with the respective doses equivalent to 500 mg/m^2, from stock pyrethrum-formulation EC and

permethrin EC based on the formula described in section 3.2.1. The nets were then carefully soaked in a non-absorbent plastic bucket containing the mixed concentrate in a 'dip-it-yourself' manner at KEMRI-CGHR. The fingers were protected with plastic gloves and impregnation properly executed in order to ensure uniform distribution of the solution onto the net. The nets were then left to dry for one-day on a glass surface in a room away from direct sunlight under ambient temperature. To assess the quality of impregnation, two pieces of net measuring 10 cm^2 were cut from different points and presented for chemical analysis using a high performance liquid chromatography (HPLC) at the Pyrethrum Board of Kenya (PBK) chemical laboratory as explained in section 3.2.1 and only nets that had a minimum of 95% of the target dose were incorporated in the trial.

3.5.2.4 Entomological evaluation on vector species, density and feeding behaviour

3.5.2.4.1 Baseline studies

Before distribution of nets, a baseline survey was conducted through the pyrethrum spray catch (PSC) method from 6.30a.m-10. 00a.m daily for 7 days to determine the indoor resting density of mosquitoes, species complex, and abdominal status classified as fed, unfed, gravid and half-gravid. In the PSC method, white cotton material sheets were spread on the floor of the experimental huts, initially, insecticide spraying was done from outside through the eaves and then into the huts using a hand sprayer. The doors and windows of the sprayed houses were then closed for 10 minutes to prevent any escaping mosquitoes and to allow for knockdown onto the spread sheets. The knocked down mosquitoes were collected from the sheets using forceps and put into petri-dishes containing moistened filter paper and thereafter sorted out into *An. gambiae* s.l. complex and *An. funestus* group using external morphological features (White *et al.*, 1972). The mosquitoes were then taken to the laboratory where female mosquitoes were sorted out into different vials individually and stored at –20°C until required.

Further identification of the *An. gambiae* s.l. was done using PCR in order to determine the identity and proportion of the prevailing sibling species in the study area. This was performed by taking a sample of N=100 of the female *An. gambiae* s.l. from the field and kept at –20°C in the laboratory as described in 3.6.3. and laboratory results on species were expressed as proportions of the total numbers analyzed (N=100).

3.5.2.4.2 Post-intervention entomological studies

After distribution of the nets, entomological surveillance continued through the pyrethrum spray catches (PSC) method. The PSC mosquito collection was conducted from 6.30 am – 10.00 am every 2 weeks for 16 weeks. The mosquitoes collected from the sheets were put in plastic cups and provided with cotton wool soaked in 10% glucose for later identification as either *An. gambiae* s.l. or *An. funestus* based on morphological features (White *et al.*, 1972) and their physiological condition such as fed, unfed, half-gravid. Persistence of the formulations on the treated nets under field use conditions was assessed by means of contact bioassays using WHO cones. The bioassays were conducted on the nets at monthly interval during the six months experimental period. In the bioassay, the cone was attached at one site of pyrethrin, permethrin and untreated net and 20 laboratory bread susceptible *An. gambiae* s.s aged 3-5 days were introduced for 3 minutes and then removed into paper cups and provided with 10% glucose soaked in cotton wool. Mortality was assessed at 24 hours. Three replicate tests were conducted per treatment.

3.6 Data Analysis

Data on counts were expressed in proportions and where the coefficient of variation was more than 15%, the data was transformed into logarithms or to arcsine [ASIN(SQRT(n)] to normalize the distribution and stabilize the variance of the data ((McDonald, 2008). Analysis of variance (ANOVA) of a randomized block design was carried out to partition variation into components for dose, time, number of washes, fabrics, colours, insecticides, houses, total mosquito density, fed, unfed, half-gravid, gravid and treatment using SAS software (SAS Institute, 1985). In cases where ANOVA table showed significant variations between treatments, least

significant difference test ($t\alpha/_2$ $S\sqrt{2/n}$) at probability level of 0.05 (LSD, $P<0.05$) was used to compare means among treatments. Dose-response data was analyzed using R version 2.11.0 statistical package. Since the relationship between the number responding (not percent response) and concentration was not normally distributed, a logit (i.e. log-logistic) analysis was used to study the insecticide dose-response relationship (Finney, 1971). The logit-regression output was used to determine the various doses that resulted in 50% knockdown (KD_{50}) and 95% knockdown (KD_{95}), while selectivity or heterogeneity indices were used for comparison of the relative potency at doses that resulted in 50% (LD_{50}).

Models of quadratic regression tests were also generated to quantify the effect of the independent variables (insecticide dosage) on the dependent variables (knockdown and kill levels), based on coefficient of determination (r^2) values and lethal doses derived from the significant trend curves or lines (Neter *et al.*, 1996). The ANOVA tests were conducted to establish the effect of pyrethrum-formulation and control insecticides on mosquitoes with *kdr* resistance genes. Differences in resistance levels between the insecticides was tested using LSD at $P=0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Efficacy of nets treated with natural pyrethrum formulation on knockdown and mortality of *An. gambiae* s.s.

Table 2 presents data on efficacy of nets treated with natural pyrethrum formulation on knockdown (KD) and mortality of *An. gambiae* s.s. ANOVA results revealed that, impregnation dose and time post-exposure were highly significant (P=0.0001) sources of variation in the levels of KD and mortality of the test mosquitoes realized that ranged from 7% KD and 17% mortality at 75mg/m^2 to 82% KD and 96% mortality at 1000mg/m^2 within 3 minutes after exposure (Table 2). This further demonstrated that there was a general increase in mortality and knockdown with increase in dose. In addition, there was a highly significant (P=0.0001) interaction between dose and time post-exposure of mosquitoes to the treated net on knockdown and mortality. For instance, at 3 minutes post-exposure, the lowest doses of 75 mg/m^2 and 100 mg/m^2 attained similar KD to that of the untreated nets, however, as the post-exposure time increased, there was a concomitant significant increase in KD for the different treatment concentrations (P=0.0001). In terms of mortality, there were highly significant (P=0.0001) differences between the 75 mg/m^2 and 100 mg/m^2. However, mortality was comparable between pyrethrins concentrations at 250 mg/m^2 and 500 mg/m^2 but with 250mg/m^2 being significantly (P=0.0001) lower than 1000 mg/m^2. At 500 mg/m^2 there was comparable mortality with 1000 mg/m^2. The positive control, permethrin performed as well as pyrethrins at the same dosage of 500mg/m^2 by realizing 92% and 90% mortality, respectively (Table 2).

Table 2: Interaction between doses of natural pyrethrum formulation (EC)- impregnated on a net and time post exposure on knockdown and kill of *Anopheles gambiae* s.s.

Conc. (mg/m^2)	%KD 3 min	%KD 5 min	%KD 10 min	%KD 15 min	%KD 30 min	%KD 60 min	%Mortality
1000	$82^{a} \pm 2.6$	$93^{a} \pm 1.0$	$95^{a} \pm 1.0$	$95.0^{a} \pm 1.0$	$97^{a} \pm 1.9$	$98^{a} \pm 1.2$	$96^{a} \pm 1.6$
500 (P)	$53^{b} \pm 3.0$	$70^{b} \pm 4.8$	$79.8^{b} \pm 0.4$	$92^{a} \pm 2.3$	$94^{a} \pm 2.6$	$94^{a} \pm 2.6$	$92^{a} \pm 0.25$
500	$48^{bc} \pm 1.5$	$67^{b} \pm 6.8$	$84.0^{b} \pm 2.1$	$94^{a} \pm 2.6$	$93^{a} \pm 3.4$	$95^{a} \pm 3.8$	$90^{ab} \pm 4.8$
250	$40.25^{c} \pm 1.3$	$49^{c} \pm 1.0$	$64^{c} \pm 3.7$	$74^{b} \pm 2.0$	$79^{b} \pm 2.5$	$84^{b} \pm 1.6$	$82^{b} \pm 2.6$
100	$10^{d} \pm 1.2$	$23^{d} \pm 1.9$	$31^{d} \pm 3.4$	$35^{c} \pm 2.5$	$37^{c} \pm 1.9$	$42^{c} \pm 2.6$	$38^{c} \pm 2.6$
75	$7^{d} \pm 1.9$	$9^{e} \pm 1.9$	$10^{e} \pm 1.2$	$10^{d} \pm 1.2$	$11^{d} \pm 1.9$	$13^{d} \pm 2.5$	$17^{d} \pm 2.5$
0	$0^{d} \pm 0.0$	$0^{e} \pm 0.0$	$0^{f} \pm 0.0$	$0^{e} \pm 0.0$	$0^{e} \pm 0.0$	$0^{e} \pm 0.0$	$0^{e} \pm 0.0$
LSD (P=0.05)	10.079	9.17	7.89	5.25	6.47±	6.76	8.12
P-Value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Data in the table are results showing the interaction effect of concentration of pyrethrum formulation- impregnated on nets (mg/m^2) and time post exposure (%KD-3min-%KD-60min) on % KD and % mortality of the test mosquitoes expressed as means ± SE . Concentration (conc/mg/m^2) refers to the amount of pyrethrum formulation-impregnated on nets expressed in mg/m^2 except the standard permethrin EC at

500mg/m^2 designated as 500P. Data in columns show the proportion (mean ± SE) of mosquitoes that were knocked down after 3-60 minutes respectively, following exposure to pyrethrum formulation-treated nets for 3 minutes. Percentage (%) mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05. *P*-values represent the calculated statistic probability levels.

4.2 Dose-response curves and determination of lethal doses and effective/optimum dose on knockdown and kill

Figure 3 presents the computed dose-response curves for each time after exposure of the mosquitoes to the treated nets together with the plotted data points as derived from the logit regression output data. The figure show a general increase in rate of change in knockdown and kill as post-exposure time increased, however this knockdown response reduced between 15-60 minutes resulting in a cluster of the respective curves (Figure 3). Taking LD_{50} as a base, there was no significant difference between KD-15 min and KD-30 (P= 0.6312), KD-15min and KD-60min (P=0.1590), KD-30min and KD-60min (P=0.3400). There was highly significant likelihood ratio (X^2 = 33.23, df=14, P=0.00266) implying that there was significant time effect. The KD_{50}, and KD_{95} at 60 minutes post-exposure was determined as 124 mg/m^2 (95% CI=108.6-139.9), and 353mg/m^2 (95% CI=221.5-484.4), respectively. The LC_{50} and LC_{95} was determined as 123.9 mg/m^2 (95% CI=105.2-142.6) and 380.71 mg/m^2 (95% CI=195-565), respectively (Table 3).

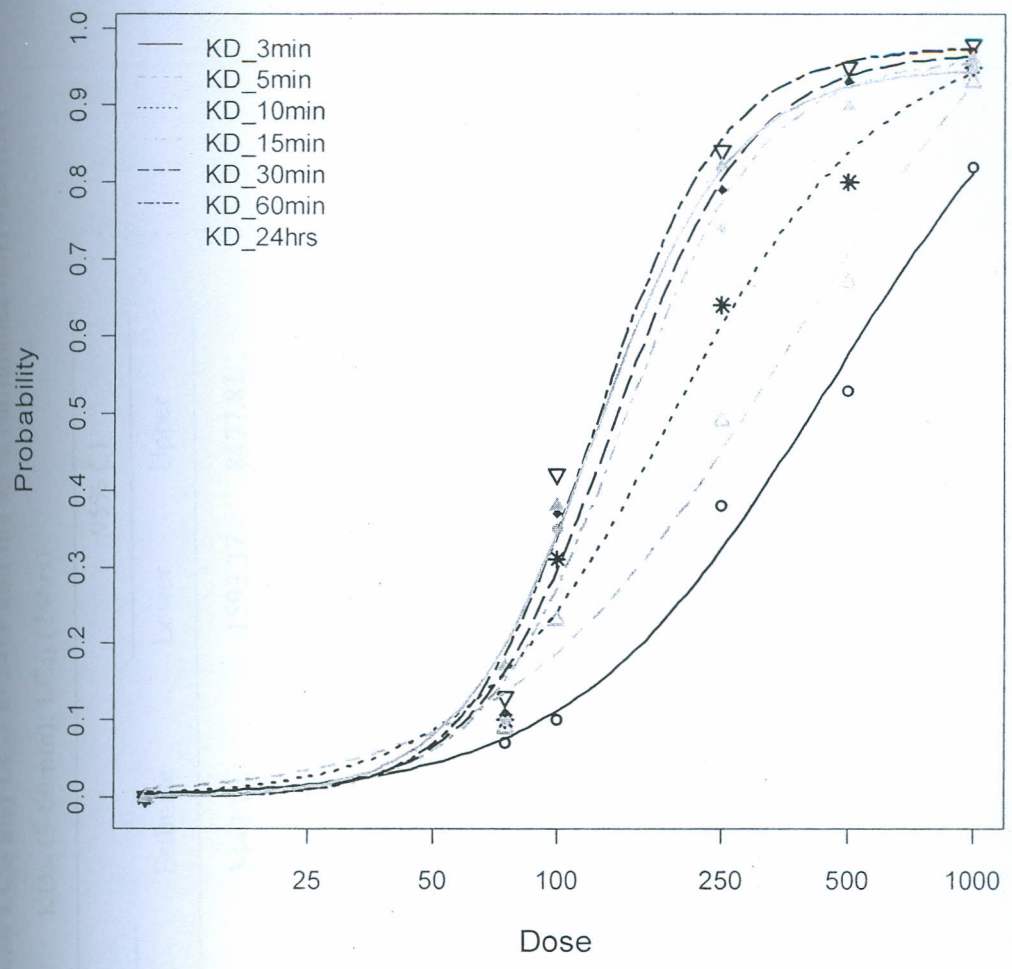

Figure 3: Dose-response curves for each post-exposure time

*Dose (mg/m^2) refers to the amount of pyrethrum formulation-impregnated on a net. Probability refers to values of probability corresponding to different % knockdown and mortality of the test mosquitoes generated from logit regression programme. The legends KD-3min, KD-5min, KD-10min, KD-15min, KD-30min and KD-60 shows the period of observation of mosquito knockdown after exposure for three minutes to various doses of pyrethrum impregnated nets.

Table 3: Estimated KD_{50} and KD_{95} for each time point and LC_{50} and LC_{95} at 24hr and the 95% confidence intervals (CI)

	KD_{50} (3-60min); LC_{50} (24hrs)			KD_{95} (3-60min); LC_{95} (24hrs)		
KD-time	Estimate	95% CI		Estimate	95% CI	
		Lower	Upper		Lower	Upper
3-min	443.64	123.55	763.72	3442.72	-1592.37	8477.81
5min	359.23	137.25	581.22	3501.92	-1422.64	8426.49
10min	188.94	142.22	235.66	1004.4	302.2	1706.6
15min	143.39	123.78	163.00	453.06	290.33	615.79
30min	136.57	117.71	155.42	419.55	257.46	581.64
60min	124.28	108.64	139.92	352.99	221.53	484.44
24hr-kill	123.91	105.2	142.62	380.71	195.56	565.86

*KD-time refers to the time post-exposure to treated net. KD_{50},and KD_{95} refers to the calculated dose of natural pyrethrum formulation-impregnated on a net that achieved 50% and 95% knockdown of the mosquitoes while LC_{50} and LC_{95} refers to the lethal concentration of the natural pyrethrum formulation-impregnated on nets that achieved 50% and 95% mortality at 24 hours post-exposure to natural pyrethrum formulation-treated nets while KL-24 refers to mosquitoes killed after 24 hours post-exposure. CI is the confidence interval of the estimates at 95% probability level.

4.3 Effect of different doses of pyrethrum formulation impregnated onto different netting fabrics on bio-efficacy against *An. gambiae* s.s.

The ANOVA results on the main effects showed that, both fabrics and insecticide dose, were highly significant (P=0.0001) sources of variation in levels of knockdown and mortality of mosquitoes realized during the tests. A highly significant interaction between dose and fabric (P=0.0001) in effecting different levels of mosquito knockdown and mortality was also observed.

The pyrethrins-impregnated cotton nets showed significantly lower perfomance (P=0.0001) relative to polyester and nylon fabrics and multiple comparisons using LSD revealed that there was significantly higher knockdown and kill with the nylon than polyester (P=0.0001, Table 4). For instance, at 30 minutes post-exporsure, nylon achieved the highest KD of 90.4% relative to 73% with polyester and only 22% with cotton. The trend was similar with mortality of mosquitoes in the treated nylon fabric, though there were lower figures for overall mortality of 61% in the treated polyester net and only 14.1% in the treated cotton fabric (Table 4).

Table 4: Effect of fabrics impregnated with natural pyrethrum formulation on mean knockdown and mortality of *Anopheles gambiae* s.s.

Fabric	% KD-30 min ± SE	% KD-60 min ± SE	% Mortality ± SE
Nylon	90.41 ± 2.06[c] (1.256)	90.41 ± 2.06[c] (1.256)	90.41 ± 2.06[c] (1.256)
Polyester	73.57± 1.62[b] (1.031)	78.26 ± 1.79[b] (1.086)	61.03 ± 1.79[b] (0.897)
Cotton	22.26 ± 0.93[a] (0.491)	45.63 ± 1.09[a] (0.742)	14.10 ± 0.69[a] (0.3849)
LSD (*P*=0.05)	0.0862	0.0604	0.0864
P-Value	0.0001	0.0001	0.0001

*Data in the table are means (± standard error) unless otherwise stated. Fabric refers to the three netting materials of nylon, polyester and cotton that were impregnated with natural pyrethrum formulation. Data in columns represented by %KD-30 and %KD-60 minutes show the proportions of mosquitoes that were knocked down after 30 and 60 minutes, respectively, following exposure to pyrethrum formulation treated nets for 3 minutes. Percentage (%) mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Figures in brackets represent % values transformed into arcsine values. Means in same column with same superscript letter are not significantly different

according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the statistic probability levels.

In addition, there was a corresponding increase in knockdown and mortality of mosquitoes with increase in the dose of impregnated pyrethrins (Table 5). Knockdown and kill of > 90% of the mosquitoes was achieved when the nets were impregnated with pyrethrins at 1000 mg/m^2 and 500mg/m^2 and these were significantly higher (P=0.0001) than at all the other treatment doses. At 500 mg/m^2, the pyrethrins-impregnated nets performed as well as the standard permethrin-treated nets at the recommended dose of 500 mg/m^2 with no apperent bioefficacy effect with the untreated nets (Table 5).

Table 5: Effect of different doses of natural pyrethrum formulation impregnated on nets on knockdown and kill of *Anopheles gambiae* s.s mosquitoes

Conc. (mg/m^2)	% KD-30 min ± SE	% KD-60 min ± SE	% Mortality ± SE
0	0.00 ± 0.00^a	0 .00 ± 0.00^a	0 .00 ± 0.00^a
100	64.61 ± 3.24^b (0.934)	83.71 ± 1.29^b (1.155)	42.93 ± 3.79^b (0.715)
250	80.78 ±1.74^c (1.117)	82.56 ± 1.05^b (1.140)	73.17± 2.43^c (1.0263)
500	83.89 ± 1.73^{c,d} (1.158)	94.31 ± 1.07^b (1.329)	81.64 ± 1.76^{c,d} (1.128)
500p	90.28 ± 1.02^d (1.254)	96.36 ± 0.68^{b,c} (1.379)	94.19± 0.6^d (1.327)
1000	97.8 ± 0.40^d (1.422)	99.68 ± 0.14^d (1.154)	95.65 ± 0.81^d (1.361)
LSD (*P*=0.05)	0.1113	0.078	0.1115
P-value	0.0001	0.0001	0.0001

*Data in the table are means (± standard error). Data in columns represented by %KD-30 and %KD-60 minutes show the proportion of mosquitoes that were knocked down after 30 and 60 minutes, respectively, following exposure to pyrethrum formulation-treated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Figures in brackets represent % values transformed into arcsine values. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic probability levels.

ANOVA results further demonstrated a significant (P=0.0001) interaction between the dosage of natural pyrethrum formulation impregnated on a net and the material of the treated fabric (Table 6). However, for the impregnated nylon fabric, there was total efficacy of 100% kill and knockdown at all the tested doses (Table 6). The results further demonstrated that the level of efficacy of a given dose was observed to be dependent on the fabric impregnated. For instance, at 30 minutes post-treatment, a polyester fabric impregnated with equivalent dose of 100 mg/m^2 of pyrethrins achieved 75% knockdown. This level was not significantly different from 250 mg/m^2 which registered 87.3% knockdown, 500 mg/m^2 that achieved 94.2% knockdown on the same fabric and 1000 mg/m^2 that effected 81.4% knockdown on natural pyrethrum formulation-impregnated cotton fabric (LSD=0.455, P=0.0001). In terms of mortality, polyester net treated at 100 mg/m^2 achieved 29.6% mortality, which was significantly different from 250-1000 mg/m^2 impregnated on cotton fabric (LSD=0.467, P= 0.0001, Table 6).

Table 6: **Interaction between natural pyrethrum formulation dose and type of impregnated netting fabrics on mean knockdown and kill of *Anopheles gambiae* s.s.**

Fabric	Conc. (mg /m^2)	KD(%)-30min ±SE	KD(%) -6min ± SE	Mortality(%) ± SE
Polyester	0	0.00 ± 0.0 ^{a}	0.00 ± 0.0^{a}	0.00 ±0.0^{a}
	100	75.00±0.06^{d,e} (1.0488)	86.22 ± 1.64^{c,d,e,f} (1.1905)	29.44 ± 0.16^{b,c} (0.574)
	250	87.34 ± 1.69^{d,e,f} (1.207)	79.30 ± 0.25^{d,e,f} (1.207)	78.71 ±4.45^{d,e,f,g} (1.0912)
	500	94.24 ± 0.8^{e,f} (1.3285)	100.00 ± 0.0^{f} (1.570)	89.97± 1.6^{d,e,f,g,h} (1.249)
	500P	94.98 ±1.95^{e,f} (1.345)	100.00 ± 0.0^{f} (1.570)	97.42 ± 0.85^{e,f,g,h} (1.4095)
	1000	100.00 ± 0.00^{f} (1.570)	100.00 ± 0.0^{f} (1.570)	100 ± 0.00^{h} (1.570)
Cotton	0	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}
	100	3.28 ± 3.28^{a,b} (0.1823)	42.20± 0.22^{b} (0.706)	0.00 ± 0.00^{a}
	250	29.55 ± 0.3^{b,c} (0.574)	46.61 ± 0.06^{b} (0.752)	16.46 ± 0.11^{a,b} (0.418) ^{b,c}
	500	29.55 ± 0.3^{b,c} (0.575)	56.42 ± 0.32^{b,c} (0.849)	28.69± 0.29^{c,d} (0.565)
	500P	56.07 ± 0.23^{c,d} (0.8462)	70.47 ± 0.26^{b,c,d,e} (1.0025)	71.03 ± 0.05^{c,d,e,f} (1.0025)
	1000	81.4 7 ± 0.06^{d,e,f} (1.126)	97.15 ± 0.96^{e,f} (1.401)	65.38 ± 0.18^{c,d,e} (0.941)
Nylon	0	0.00 ± 0.0^{a}	100.00 ± 0.0^{f} (1.570)	0.00 ± 0.0^{a}
	100	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{h} (1.570)
	250	100.00-± 0.0^{f} (1.570)	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{h} (1.570)
	500	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{h} (1.570)
	500P	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{h} (1.570)
	1000	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{f} (1.570)	100.00 ± 0.0^{h} (1.570)
LSD		0.455	0.438	0.467
P-Value		0.0001	0.0001	0.0001

*Data in the table are means (± standard error). Fabric refers to the three netting materials of nylon, polyester and cotton that were impregnated with natural pyrethrum–formulation. Conc. refers to the

concentration of natural pyrethrum formulation that was impregnated onto a net. Data in columns represented by KD(%)-30 and KD(%)-60 minutes show the proportions of mosquitoes that were knocked down after 30 and 60 minutes, respectively, following exposure to natural pyrethrum formulation-treated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Figures in brackets represent % values transformed into arcsine values. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the statistic probability levels.

To further determine the quantitative effect of the treated fabric and impregnated dose of the natural pyrethrins formulation on mosquito knockdown and mortality, regression tests were conducted and results are shown in Table 7. The results show that, the effect of impregnated dose on knockdown and kill was not linear, but rather quadratic in nature as observed from the coefficient of determination (r^2) values. The dose-response model in cotton fabric had relatively higher r^2 values of more than 0.9 as compared the same polyester fabric which had a r^2 value of more than 0.75. However, there was relatively lower r^2 values for the nylon nets.

Regression coefficients which describe the effect of unit increase in dose on knockdown and kill of the test mosquitoes are also presented in Table 7. At the linear portion of the quadratic equation, there was higher increase per unit dose in treated nylon and polyester fabrics, than in cotton fabric. For example, at 30 minutes post-exposure to the pyrethrum formulation-impregnated nets, a unit increase in dose resulted in 0.291% ($y=32.53 + 0.291x-0.000229x^2$) increase in knockdown in nylon nets, 0.277% ($y=21.67 + 0.277x-0.00021x^2$) in polyester net and only 0.058% ($y=4.33 + 0.058x-0.0000174x^2$) in cotton fabric . However, after the linear portion of the quadratic equation, there were subsequent negative regression coefficients although

the decrease was again higher in nylon netting fabric than in polyester and lowest in cotton (Table 7).

Table 7: Regression models of dose of natural pyrethrum formulation impregnated on nets against knockdown and kill of *Anopheles gambiae* s.s.

Fabric	Regression equation (y = KD(%)-30min, x = mg/m^2)	r^2
Polyester	$y=21.67 + 0.277x - 0.00021x^2$	0.78
Cotton	$y = 4.33 + 0.058x - 0.0000174x^2$	0.96
Nylon	$y = 32.53 + 0.291x - 0.000229x^2$	0.59
	Regression equation (y = KD(%)-60min, x = mg/m^2)	
Polyester	$y=23.83 + 0.283x - 0.00021\ x^2$	0.75
Cotton	$y=12.94 + 0.140x - 0.000124\ x^2$	0.88
Nylon	$y=32.53 + 0.291x - 0.291\ x^2$	0.51
	Regression equation (y = mortality(%), x = mg/m^2)	
Polyester	$y=4.63 + 0.269x-0.00017\ x^2$	0.75
Cotton	$y=0.04+ 0.15x-0.000051\ x^2$	0.99
Nylon	$y=32.53 +-0.29-0.000229\ x^2$	0.59

*Fabric refers to the three netting materials, nylon, polyester and cotton that were impregnated with natural pyrethrum formulation. Regression equations are the models that describe the unit effect of the independent variable, concentration (X) and dependent or response variables, knockdown (KD (%)-

30min, KD(%)-60min) and mortality depicted by y. The r^2 represents the coefficient of determination showing what quantity of variation is explainable by the equation.

The non-linearity of the dose-response observed in the following work shows that, increase in dose of pyrethrum formulation impregnated on a net is not directly proportionate to the increase in bio-efficacy, with higher unit increase per dose realized in natural pyrethrum formulation-impregnated nylon, and with the least increase in treated cotton fabric. The dose of natural pyrethrum formulation that achieved 95% knockdown of mosquitoes (Kd_{95}) after 60 minutes post initial three minutes exposure on treated polyester fabric was 350 mg/m^2 and 275mg/m^2 on nylon (Figure 4). In terms of effecting the standard 80% mortality (LC_{80}) after 24 hours, polyester fabric required 375 mg/m^2 compared to only 200 mg/m^2 for nylon fabric (Figure 5). In contrast, cotton fabric required a much higher dose of insecticide in order to achieve the lethal values (Figures 4 and 5).

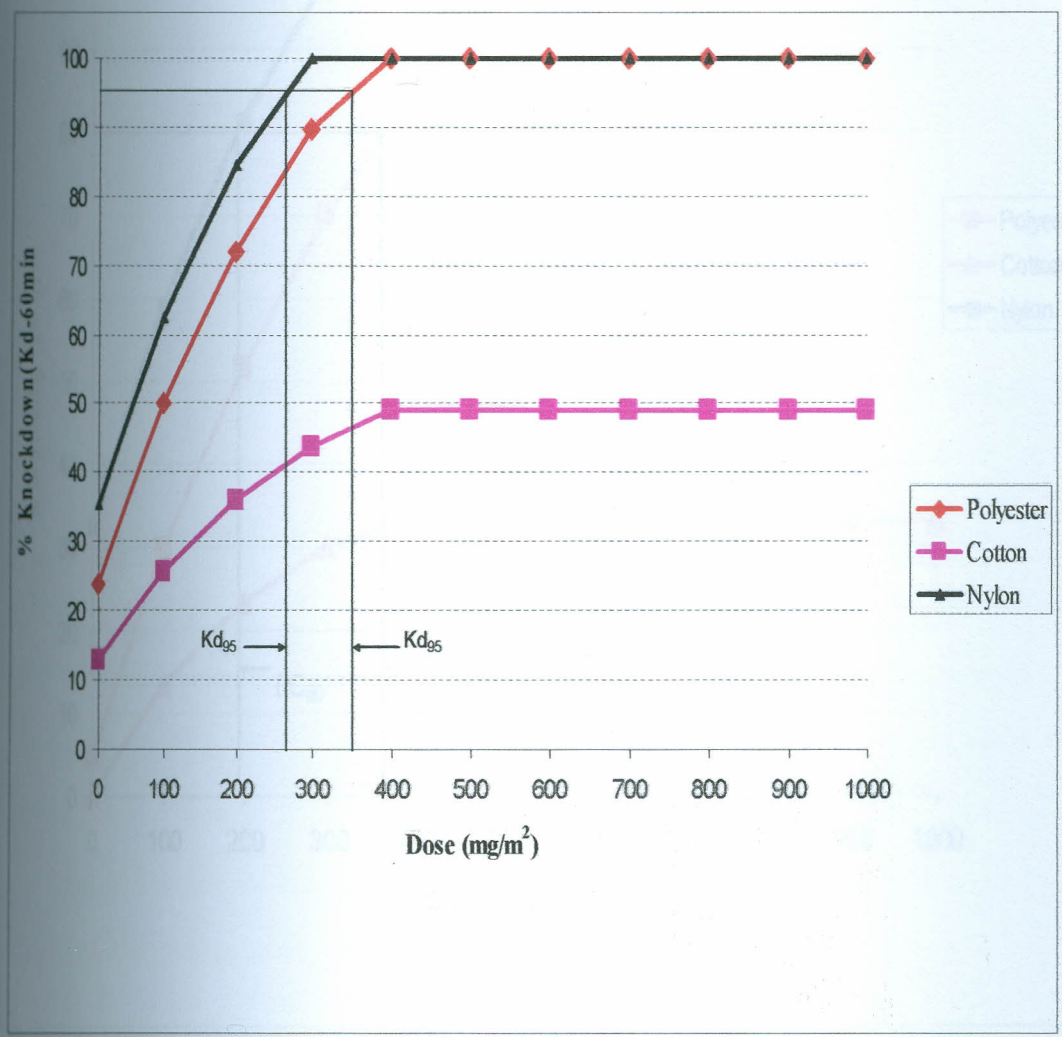

Figure 4: Predicted knockdown of *Anopheles gambiae* s.s. exposed to natural pyrethrum formulation-treated netting fabrics at different doses.

*Three netting materials nylon, polyester and cotton were impregnated with pyrethrum formulation. Kd_{95} refers to the calculated dose of natural pyrethrum formulation-emulsifiable concentrate that achieved 95% knockdown of mosquitoes after 60 minutes following exposure of mosquitoes to pyrethrins-treated nets initially for 3 minutes. For the polyester fabric, it was extrapolated to be 350 mg/m^2 based on the regression equation $y = 4.63 + 0.269x - 0.00017x^2$ and for nylon it was 275 mg/m^2 $y = 32.53 + -0.29 - 0.000229x^2$ where **y** is predicted % knockdown and **x** is concentration of pyrethrum-impregnated on a net in mg/m^2.

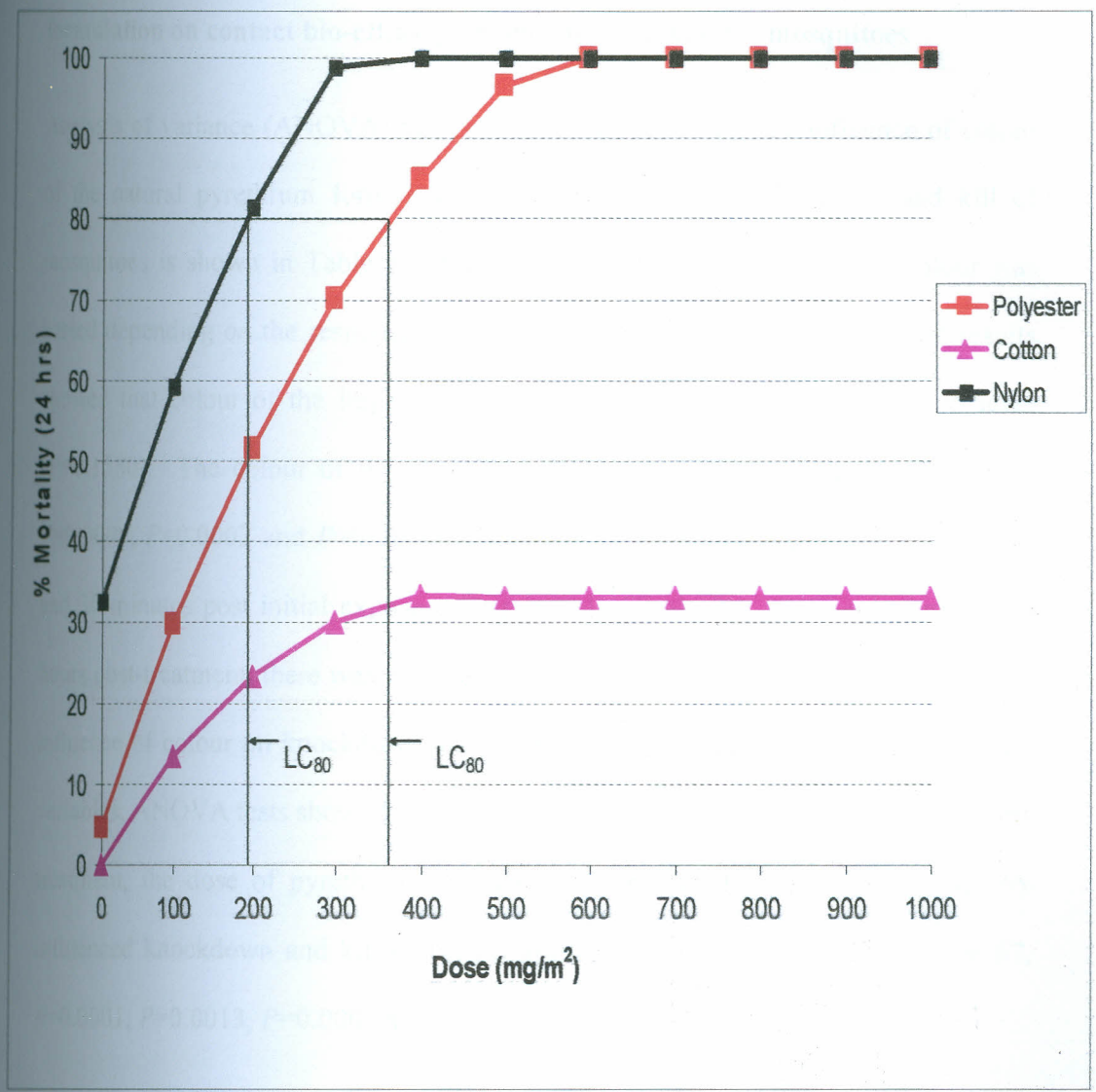

Figure 5: Predicted mortality of *Anopheles gambiae* s.s. exposed to natural pyrethrum formulation treated-netting fabrics at different doses.

*The three netting materials, nylon, polyester and cotton were impregnated with natural pyrethrum formulation. LC_{80} is a calculated dose that achieved the standard 80% kill of test mosquitoes after 24 hours following 3 minutes exposure to pyrethrum formulation-treated nets. For polyester fabric, it was 375 mg/m^2 extrapolated from the regression equation $y = 4.63 + 0.269x - 0.00017x^2$ and for nylon it was 200 mg/m^2 based on regression equation $y = 32.53 + 0.29 - 0.000229x^2$ where y is predicted % mortality and x is concentration of pyrethrum-impregnated on a net in mg/m^2.

4.4 Effect of colour of netting fabric impregnated with natural pyrethrum formulation on contact bio-efficacy against *An. gambiae* s.s. mosquitoes

Analysis of variance (ANOVA) tests conducted to determine the influence of colour of the natural pyrethrum formulation-impregnated net on knockdown and kill of mosquitoes is shown in Table 8. It was observed that the influence of colour was varied depending on the response variable. At 3 minutes post-treatment, the results showed that colour of the impregnated net did not influence the knockdown level (P=0.1580). The colour of the impregnated net however significantly (P=0.0026, P=0.0004, P=0.0002 and P=0.0161) influenced the level of knockdown at 5, 10, 15 and 30 minutes post initial exposure, respectively (Table 8). At 60 minutes and 24 hours post-treatment, there was no significant (P=0.2910 and P=0.8342, respectively) influence of colour on knockdown and mortality of mosquitoes. In all the response variables, ANOVA tests showed that at 3, 5, 10, 15, 30, 60 minutes and 24 hours post-treatment, the dose of pyrethrum formulation-impregnated into a net significantly influenced knockdown and kill of the mosquitoes (P=0.0123, P=0.0037, P=0.0167, P=0.0001, P=0.0013, P=0.0001 and P=0.0007, respectively).

Further findings demonstrate that, there was significant interaction between colour and dose of pyrethrum formulation- impregnated on a net in effecting knockdown of mosquitoes at 15, 30 and 60 minutes post-treatment (P=0.0043, P=0. 0430 and P=0.0198, respectively, Table 8). Results also showed that there was no significant interaction between the colour of the pyrethrum formulation treated fabric and dose of pyrethrum formulation impregnated in influencing mortality (P=0.4028) and immediate knockdown at 3, 5 and 10 minutes post-treatment with pyrethrum formulation (P=0.2989, P=0.3178 and P=0.1071, respectively) (Table 8).

Table 8: ANOVA probability (P) values on effect of colour of treated net and dose of pyrethrum formulation-impregnated on knockdown and mortality of *Anopheles gambiae* s.s.

Source	KD 3min	KD 5min	KD 10min	KD 15min	KD 30min	KD 60min	% Mortality
Colour	0.1585 ^{NS}	0.0026**	0.00044***	0.0002***	0.0161**	0.2910 ^{NS}	0.8342^{NS}
Dose	0.0123*	0.0037**	0.0167*	0.0001***	0.0013**	0.0001***	0.0007***
Colour X Dose	0.2989^{NS}	0.3178^{NS}	0.1071 ^{NS}	0.0043**	0.043*	0.0198*	0.4028^{NS}

*Data in the table are ANOVA probability values showing influence of colour of a net, dose of natural pyrethrum formulation-impregnated on a net and interaction between dose and colour on mosquito knockdown at 3-60 minutes, and mortality after 24 hours post-exposure for 3 minutes to pyrethrum formulation-impregnated netting material. Levels of significance are shown as *** implying high significance at 99.99%, ** significance up to 99.7%, *shows significance up to 98.7% while NS refers to no significance at 95% level.

Additional data summarizing the overall biological performance of green, blue and white polyester coloured nets impregnated with pyrethrins is shown in Table 9. It was observed that at 3 minutes post-exposure, there were no significant differences (P=0.158) among the coloured fabrics in influencing levels of mosquito knockdown. However, at 5 and 10 minutes post-treatment, there was significantly higher knockdown in the blue and green coloured treated nets (P=0.0026 and P=0.00044, respectively) relative to the white treated net. Furthermore, at 15 and 30 minutes post-treatment, treated green net had higher knockdown level (over 90%) relative to blue (P=0.0002) and white coloured nets (P=0.0161).

These findings show that there appears to be an apparent influence of the colour of a treated net on the early knockdown of mosquitoes, however, there is no clear effect on late knockdown and mortality.

Table 9: Effect of colour of netting fabrics treated with natural pyrethrum formulation on knockdown and kill of *Anopheles gambiae* s.s.

Netting Colour	KD (%) 3min ± SE	KD (%) 5min ± SE	KD (%) 10min ± SE	KD (%) 15min ± SE	KD (%) 30min ± SE	KD (%) 60min ± SE	Kill(%) ± SE
White	61.68[a] ± 0.11 (0.9033)	61.74[a] ± 0.18 (0.9039)	66.86[a] ±0.16 (0.9574)	76.0[a] ± 0.27 (1.059)	85.78[ab] ± 0.15 (1.184)	89.61[a] ± 0.11 (1.243)	85.65[a] ± 0.49 (1.182)
Green	70.62[a] ± 0.35 (0.998)	73.82[b] ± 0.10 (1.034)	82.47[b] ± 0.2 (1.139)	90.90[b] ± 0.68 (1.265)	92.27[b] ± 0.56 (1.289)	93.95[a] ± 0.66 (1.322)	85.82[a] ± 0.4 (1.185)
Blue	72.78[a] ± 0.22 (1.022)	76.23[b] ± 0.06 (1.061)	75.88[ab] ± 0.10 (1.057)	75.61[a] ± 0.03 (1.054)	84.49[a] ± 0.1 (1.166)	93.41[a] ± 0.48 (1.311)	88.16[a] ± 0.72 (1.219)
P-Value	0.1585	0.0026	0.00044	0.0002	0.0161	0.2910	0.8342
LSD	0.124	0.088	0.119	0.946	0.113	0.088	0.16

*Data in the table are means (± standard error). Netting colour refers to the three polyester nets coloured white, green, and blue that was impregnated with natural pyrethrum formulation. Data in columns represented by KD (%)-3min, KD (%)-5min, KD(%)-10min, KD(%)- 15min, KD (%)-30mi, %KD(%)-60min show the proportion of mosquitoes that were knocked down after the respective minutes, following exposure to natural pyrethrum formulation- treated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post initial exposure. Figures in brackets represent % values transformed into arcsine values. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic probability levels.

Table 10 shows KD and mortality values realized based on interaction effect of the colour and dose of pyrethrum formulation-treated net. Results show that at 3, 5 and 10 minutes post-treatment, at same dose level, there was no significant interaction between colour and dose in effecting knockdown (P=0.298, P=0.317 and P=0.107, respectively). However, there was significant interaction at 15, 30, and 60 minutes post-treatment (P=0.004, P=0.040 and P=0.019, respectively). For instance, at 15 minutes post-treatment, blue nets treated with natural pyrethrum formulation at 500 mg/m^2 had a 74% mosquito knockdown, while white had 85.41 and both were significantly (P=0.004) lower than the 98.9% knockdown realized in green nets. The interaction of colour of a net and the pyrethrum formulation treatment dose however, did not significantly affect mosquito mortality (P=0.402).

These findings imply that the bio-efficacy realized by a given dose was influenced by the colour of the treated net. However, this is likely to vary with the amount of dose impregnated on a net.

Table 10: Interaction of fabric colour and dose of natural pyrethrum formulation impregnated on a net on knockdown and kill of *Anopheles gambiae* s.s.

Netting Colour	Conc. mg/m^2	KD (%) 3min ± SE	KD (%) 5min ±SE	KD (%) 10min± SE	KD (%) 15min ± SE	KD (%) 30min ± SE	KD (%) 60min ±SE	Kill(%) ± SE
White	250	58.09^{a} ± 0.18 (0.867)	54.04^{a} ± 0.29 (0.826)	60.90^{a} ± 0.19 (0.887)	65.10^{d} ± 0.13) (0.939)	83.42^{b} ± 0.19 (1.151)	85.15^{c} ± 0.07 (1.179)	78 .03^{a} ± 0.02 (1.083)
Green	250	59 .07^{a} ± 0.12 (0.877)	67.04^{a} ± 0.04 (0.959)	76.16^{a} ± 0.11 (1.061)	76.10$^{b.d}$ ± 0.04 (1.059)	80.08^{b} ± 0.04 (1.108)	81.34^{c} ± 0.19 (1.124)	74.61^{a} ± 0.5 (1.043)
Blue	250	68.16^{a} ± 0.16 (0.971)	74.13^{a} ± 0.08 (1.037)	72.12^{a} ± 0.09 (1.015)	74.03$^{c.d}$ ± 0.017 (1.037)	79. 10^{b} ± 0.06 (1.096)	83.49^{c} ± 0.23 (1.152)	72.53^{a} ± 0.43 (1.019)
White	500	65.20^{a} ± 0.22 (0.940)	69.14^{a} ± 0.135 (0.989)	73.29^{a} ± 0.24 (1.028)	85.41^{b} ± 0.19 (1.179)	87.99^{b} ± 0.43 (1.217)	93.35^{b} ± 0.14 (1.31)	91.87^{a} ± 1.6 (1.238)
Green	500	80.97^{a} ± 0.6 (1.119)	80.08^{a} ± 0.04 (1.108)	87.99^{a} ± 0.43 (1.217)	98. 99^{a} ± 0.34 (1.470)	98.99^{a} ± 0.34 (1.470)	99. 74^{a} ± 0.25 (1.520)	94.16^{a} ± 0.06 (1.327)
Blue	500	77.10^{a} ± 0.70 (1.073)	78.26^{a} ± 0.17 (1.086)	79.44^{a} ± 0.26 (1.100)	77.18$^{b.c}$ ± 0.12 (1.074)	89.21^{b} ± 0.09 (1.236)	98.99^{a} ± 0.34 (1.470)	97.74^{a} ± 0.25 (1.420)
P-value		0.2989	0.3178	0.1071	0.0043	0.0407	0.0198	0.4028
LSD (α=0.05)		0.275	0.294	0.341	0.134	0.160	0.124	0.421

*Data in the table are means (± standard error). Netting colour refers to the three polyester nets coloured. white. green. and blue that were impregnated with natural pyrethrum formulation at the two dose levels of 250 mg/m^2 and 500 mg/m^2. Data in columns represented by KD (%)-3min. KD (%)-5min KD (%)-10min KD (%)- 15min. KD (%)-30min. KD (%)-60 min show the proportion of mosquitoes that were knocked down after the respective minutes. following exposure to pyrethrum

formulation-impregnated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Figures in brackets represent % values transformed into arcsine values. Means in same column for same concentration having similar superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic probability levels.

4.5 Effect of washing on persistence of bio-efficacy of natural pyrethrum formulation treated nets

Results on wash-resistance of pyrethrum formulation-treated nets are shown in Table 11. Washing of pyrethrum formulation-impregnated bed nets significantly (P=0.001) affected knockdown and kill of mosquitoes. For instance, at the recommended treatment rate of 500mg/m^2, the unwashed nets achieved a mosquito knockdown of 99% at 60 minutes post-exposure and kill of 95% after 24 hours. This level of efficacy was comparable with the efficacy of the treated nets washed once (1X) (one wash) , which showed that at 60 minutes, there was 97% KD and 96% kill, and the nets when washed twice (2X) at 60 minutes achieved 96% KD and 93% kill after 24 hours post-exposure (Table 10). However, a significant reduction (P=0.0001) in efficacy was observed after wash three times (3X) when 85% KD and 85% kill were realized. Substantially reduced efficacy was observed on nets washed four times (4X), which only realized 46% KD and 42% kill after 60 minutes. This was further reduced to 23% KD and 17% kill after five washes (5X).

Further results from chemical analysis revealed that there was significant reduction in the amount of impregnated pyrethrins remaining on treated nets as the number of washes increased (Table 11). Results also demonstrated that there was 26%, 55%, 68%, 89% and 100% reduction in pyrethrins against the initially impregnated level after 1, 2, 3, 4, and 5 washes, respectively (Table 11).

Table 11: Effect of washing netting fabrics treated with natural pyrethrum formulation at 500mg/m^2 on knockdown and kill of *Anopheles gambiae* s.s .and residual persistence of pyrethrins treated nets

No. of Washes	KD(%) 3min ± SE	KD(%) 15min ± SE	KD(%) 30min ± SE	KD(%) 60min ± SE	Kill (%)l ± SE	% Pyrethrins on net ± SE	Quantity of pyrethrins (mg/100cm^2) ± SE
0	23.0^a ± 1.9 (4.9)	64.0^a ± 1.6 (8.1)	88.0^a ±3.7 (9.4)	99.0^a ± 1.0 (10.0)	95.0^{ab} ± 1.9 (9.7)	1.18 ± 0.02^a	5.08 ± 0.07^a
1	22.0^a ± 2.6 (4.8)	57.0^a ± 3.4 (7.6)	87.0^a ± 1.9 (9.4)	97.0^a ± 1.9 (9.9)	96^a ± 2.8 (9.8)	0.86± 0.03^a	3. 61 ± 0.13^a
2	25.0^a ± 4.4 (5.0)	50.0^a ± 4.2 (7.1)	81.0^a ± 3.0 (9.1)	96.0^a ± 1.6 (9.8)	93^{ab} ±3.0 (9.7)	0.54 ± 0.01^a	2.19 ± 0.06^a
3	6.0^b ± 1.2 (2.62)	18.0^b ± 4.2 (7.1)	35.0^a ± 10.4 (5.8)	85^b ± 1.9 (9.3)	85^b ±1.9 (9.3)	0.38 ± 0.01^b	1.49 ± 0.05^b
4	8.0^b ± 1.6 (2.9)	10.0^c ± 2.6 (3.2)	13.0^c ± 1.6 (3.6)	46^c ± 2.6 (6.8)	42^c ±3.8 (6.6)	0.14 ± 0.01^b	0.54 ± 0.04^{bc}
5	7.0^b ± 2.5 (2.7)	8.0^c ± 1.6 (3.0)	12.0^c ± 1.9 (3.7)	23.0^d ± 1.9 (4.9)	17.0^d ±1.9 (4.2)	0.01 ± 0.0$^{b.c}$	0.04 ± 0.0^c
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0002
LSD α = 0.05	7.7	9.3	14.4	5.6	7.9	0.011	0.034

*Data are means (± standard error) unless otherwise stated. Netting colour refers to the three polyester nets coloured, white, Green, and blue that were impregnated with natural pyrethrum-formulation. Data in columns represented by KD (%)-3min, KD (%)-5min, KD(%)-10min, KD(%)- 15min, KD(%)-30mi. KD(%)-60min show the proportion of mosquitoes that were knocked down after the respective

minutes, following exposure to natural pyrethrum formulation-treated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post initial exposure. Figures in brackets represent % values transformed into arcsine values. Percentage (%) pyrethrins on net show the amount of pyrethrins in the treated net determined by high performance liquid chromatography (HPLC). Quantity of pyrethrins refers to the calculated amount of pyrethrins on net samples in mg/100cm^2. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic probability levels.

4.6 Residual persistence of pyrethrins on impregnated nets

Results on bio-efficacy of unwashed treated nets over a six-month period are shown in Table 12. There was a significant difference (P=0.004) between the treated and untreated nets in terms of KD at 60 minutes post- initial exposure from month 2 after impregnation. However, significant difference (P=0.028) in mortality was observed only at 6 months post-treatment. In general, both KD at 60 minutes and mortality after 24 hours remained consistently high at above 90% over the whole six-month testing period.

Results on chemical analysis of residual pyrethrins remaining on treated nets over a 6-month period is also shown in Table 12. It was observed that time (months) post-treatment significantly (P=0.0002) affected the quantity of pyrethrins remaining on unwashed treated nets. A significant (P=0.0002) reduction in quantity of pyrethrins was observed at 3 months post-treatment, with the lowest reduction recorded in the 6 months after impregnation, when there was 3.81mg/100cm^2 (381mg/m^2) against the initial impregnated dose of 5.09 mg/100cm^2, representing a 25% reduction (Table 12).

Table 12: Residual bio-efficacy of nets treated with natural pyrethrum formulation at 500mg/m^2 on knockdown and mortality of *Anopheles gambiae* s.s. and chemical persistence of pyrethrins on nets over six months.

Month	KD(%) 3min ± SE	KD(%) 15min ± SE	KD(%) 30min ± SE	KD(%) 60min ± SE	Mortality (%) ± SE	% Pyrethrins on net ± SE	Quantity of rethrins (mg/100cm^2) ± SE
0	24.0^{cd} ± 3.2	72.0^{a} ± 2.4	90.0^{a} ±2.2	99.0^{a} ± 0.9	98.0^{a} ± 1.7	1.18 ± 0.015^{a}	5.09 ± 0.066^{a}
1	26.0^{bcd} ± 4.6	61.0^{b} ± 3.3	89.0^{a} ± 1.7	92.0^{ab} ± 1.7	96.0^{ab} ± 1.4	1.21 ± 0.022^{a}	5.15 ± 0.124^{a}
2	21.0^{d} ± 2.7	60.0^{bc} ± 3.2	86.0^{a} ± 2.2	92.0^{b} ± 1.4	97.0^{a} ± 0.9	1.19 ± 0.03^{a}	5.14 ± 0.02^{a}
3	37.0^{ab} ± 4.1	5.0^{bc} ± 5.0	87.0^{a} ± 2.2	96.0^{ab} ± 2.0	94.0^{ab} ± 2.2	1.06 ± 0.02^{b}	4.57 ± 0.104^{b}
4	40.0^{ab} ± 3.2	55.0^{bc} ± 2.6	87.0^{a} ± 3.0	95.0^{ab} ± 1.7	94.0^{ab} ±1.7	1.02 ± 0.02^{b}	4.33 ± 0.03^{bc}
5	38.0^{ab} ± 4.6	50.0^{c} ± 2.2	86.0^{a} ± 2.2	92.0^{b} ± 1.4	94.0^{ab} ± 2.2	0.96 ± 0.03^{b,c}	4.10 ± 0.104^{c}
6	34.0^{abc} ± 3.6	51.0^{bc} ± 1.7	86.0^{a} ± 2.2	92.0^{b} ± 1.4	91.0^{b} ±1.7	0.91 ± 0.025^{c}	3. 81 ± 0.07^{d}
P-value	0.023	0.0044	0.888	0.0040	0.0284	0.0001	0.0002
LSD α = 0.05	12.6	10.4	7.7	5.1	5.9	0.011	0.034

*Data in the table are means (± standard error). Month refer to the period when bioassay tests were conducted on the pyrethrum-formulation treated nets. Data in columns represented by KD (%)-3min,

KD (%)-5min, KD (%)-10min, KD (%)-15min, KD (%)-30mi, KD (%)-60min show the proportion of mosquitoes that were knocked down after the respective minutes, following exposure to pyrethrum-formulation treated nets for 3 minutes. Percent (%) mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Percent (%) pyrethrins on net shows the amount of pyrethrins in the treated net determined by high performance liquid chromatography (HPLC). Quantity of pyrethrins, refers to the calculated amount of pyrethrins on net samples in mg/10cm^2 . Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05.

4.7 Determination of the *Anopheles species* occurrence and *kdr* mutation status in Kisian, Kipsitet and Ahero areas and assess susceptibility of *Anopheles spp.* with *kdr* genes to the synergized natural pyrethrum formulation

4.7.1 Species identification by PCR

Based on the resultant gel electrophoresis images showing DNA bands for the individual mosquitoes analysed, propotions of *An. gambiae* and *An. arabiensis* identified from the samples from the three study were determined as shown in Figure 6. The figure shows that, 100% of the mosquito samples from Kipsitet and Ahero were *An. arabiensis* while in Kisian, 73% were *An. arabiensis* and 27% were *An. gambiae* s.s. Overall in the study areas, *An. arabiensis* formed 91% of the population while *An.gambiae* s.s. formed only 9%.

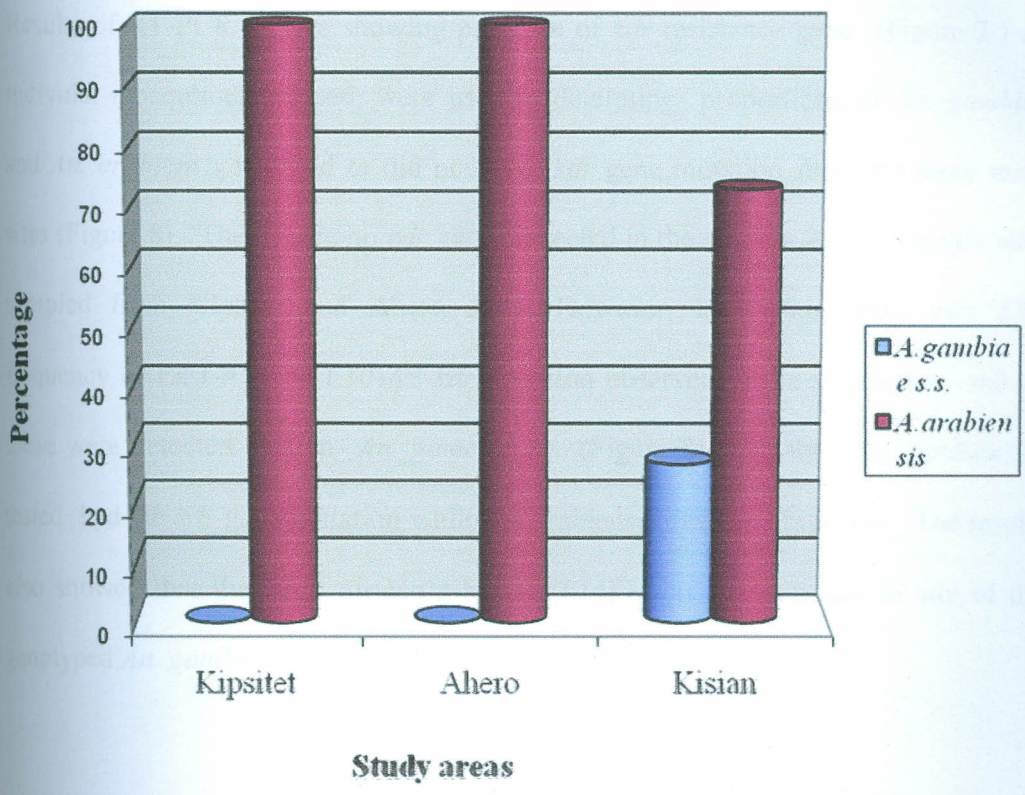

Figure 6: Comparative distribution of *Anopheles gambiae* s.l species in Kipsitet, Ahero and Kisian areas.

*Data are proportions of *An. gambiae* s.s. and *An. Arabiensis* collected from Kipsitet, Ahero and Kisian study sites as determined from the PCR results.

4.7.2 Detection of *kdr* gene mutations using RT-PCR

Results of RT-PCR images showing presence of *kdr* resistance gene (Figure 7) on indivulal mosquitoes anlysed, were used to determine proportions of *An. gambiae* and *An. arabiensis* that had or did not have *kdr* gene mutation from the three study sites (Figure 8). There were no *kdr* genes detected in the *Anopheles* species that were sampled from Kipsitet and Ahero areas. However, in Kisian, there was 27% frequency of East African L1014S *kdr* mutation observed in the population and all these were detected only in *An. gambiae* s.s. (Figure 8). All the *An. gambiae* s.s tested had the *kdr* gene mutation while An arabiensis did not. (Figure9). The results also showed that the West african allele (L1014F) was not detected in any of the genptyped *An. gambiae* s.l. individuals from the three study sites.

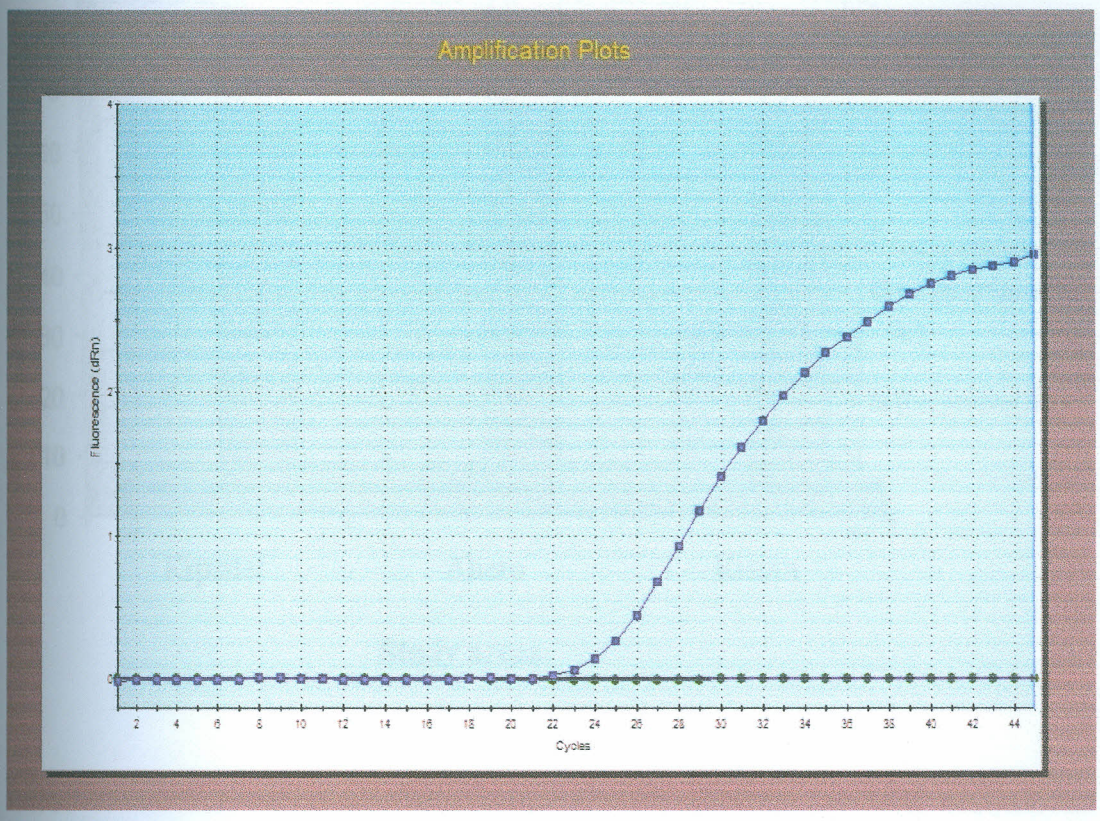

Figure 7: RT-PCR output showing presence of *kdr* mutation in a mosquito sample.

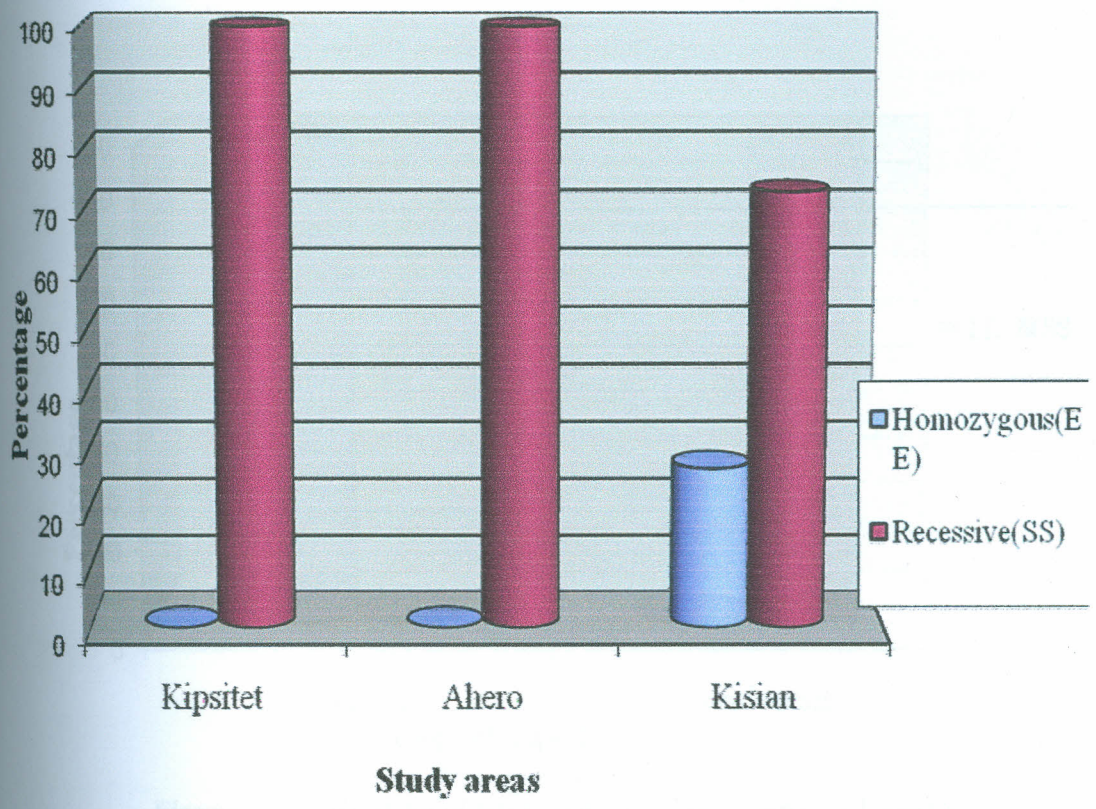

Figure 8: Distribution of *kdr* resistance genes in Kipsitet, Ahero and Kisian areas.

*Homozygous (EE) refers to the *Anopheles* mosquitoes that had East Africa L1014S *kdr* resistance genes while SS refers to susceptible mosquitoes recorded in the three study sites of Kipsitet, Ahero and Kisian.

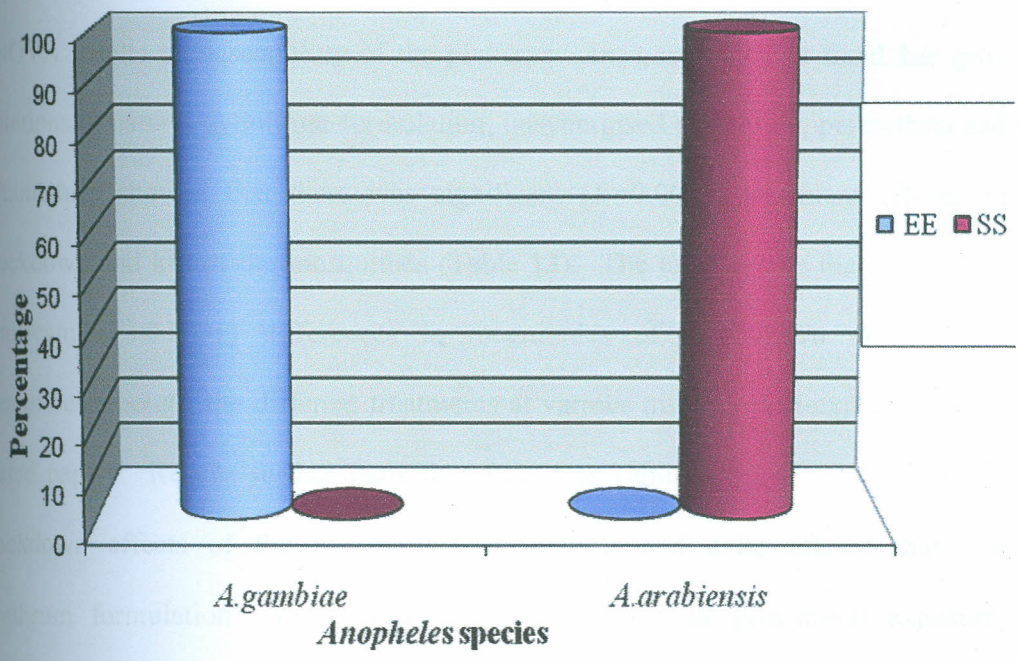

Figure 9: Distribution of *kdr* mutation in *An. gambiae* s.l. species

*Homozygous (EE) refers to the East Africa L1014S *kdr* resistance genes while SS refers to susceptible mosquitoes recorded in *An. gambiae* s.s and *An. arabiensis* collected from the three study sites.

4.7.3 Effect of natural pyrethrum formulation on pink-eyed strain *Anopheles gambiae* s.s. with fixed L1014S *kdr* gene mutation

ANOVA results on susceptibity of the pink-eyed *An. gambiae* with fixed *kdr* gene mutations to natural pyrethrum formulation, unsynergised pyrethrins, permethrin and deltamethrin showed that there was significant (P=0.0001) treatment effects on knockdown and kill of the mosquitoes (Table 13). The table shows that there were significant (P=0.0001) differences in magnitudes of knockdown and kill of mosquitoes amongst the different treatments at various minutes post-exposure to the treated papers. Results further show that there was significantly (P=0.0001) lower knockdown effects of the pyrethroids (permethrin and deltamethrin) than the pyrethrum formulation although from 15 min – 1 hour post-initial exposure, deltamethrin achieved significantly (P=0.0001) higher knockdown than permethrin. For instance, at 60 minutes, permethrin had only 6% KD compared to 43% KD for deltamethrin and 100% KD for pyrethrum formulation. It was also observed that there was no significant (P=0.05) difference between the natural pyrthrum formulation and the unsynergised pyrethrum in effecting knockdown of mosquitoes.

Regarding mortality, Table 13 further shows that natural pyrethrum formulation achieved significantly (P=0.0001) higher kill of 98.7% as compared to 87% with deltamethrin, and 88% with unsynergized pyrethrins even though the latter two treatments were not significantly (P=0.05) different. In all the instances, permethrin had significantly (P=0.0001) lower kill effect of only 12.5% as compared to the other treatments.

Table 13: Comparative susceptibility of pink-eyed *An gambiae* s.s. with *kdr* genes to natural pyrethrum-formulation, technical grade pyrethrum, permethrin and deltamethrin

Treatment	KD(%) 3min ± SE	KD(%) 5min ± SE	KD(%) 10min ± SE	KD(%) 15min ± SE	KD(%) 30min ± SE	KD(%) 60min ± SE	Mortality (%) ± SE
Permethrin 0.75%	0.0^{b} ± 0.0	0.0^{b} ± 0.0	1.25^{bc} ± 1.25	1.25^{c} ± 1.25	6.23^{c} ±2.4	6.25^{c} ± 3.1	12.5^{c} ± 1.4
Deltamethrin 0.05%	1.25^{b} ± 1.25	2.5^{b} ± 1.4	8.75^{b} ± 1.2	15.0^{b} ± 1.2	22.5^{b} ± 8.7	43.75^{b} ± 6.3	87.5^{b} ± 3.2
Pyrethrum formulation 1% (synergized)	70.0^{a} ± 10.2	80.0^{a} ± 5.0	90.0^{a} ± 2.0	97.5^{a} ± 1.4	100^{a} ± 0.0	100^{a} ± 0.0	98.75^{a} ± 1.3
Pyrethrum extract 1% (unformulated)	82.5^{a} ± 6.1	83.7^{a} ± 2.4	91.25^{a} ± 2.4	96.2^{a} ± 2.4	98.75^{a} ± 1.25	96.25^{a} ± 1.25	88.75^{b} ± 3.2
Untreated	0.0^{b} ± 0.0	0.0^{b} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{c} ± 0.0	0.0^{d} ±0.0
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
LSD (α = 0.05)	14.52	9.07	8.31	8.8	12.4	9.58	7.15

*Data in the table are means (± standard error) . Month refer to the period when bioassay tests were conducted on the pyrethrins-treated nets. Data in columns represented by KD (%)-3min, KD (%)-5min, KD (%)-10min KD (%)-15min, KD (%)-30min, KD (%)-60min show the proportion of mosquitoes that were knocked down after the respective minutes, following exposure to pyrethrins-treated nets for 3 minutes. % mortality refers to the proportion of mosquitoes that died after 24 hours post exposure. Perecent (%) pyrethrins on net shows the amount of pyrethins that was contained in the treated test papers.

4.8 Susceptibility of wild *An. gambiae* s.l. from Ahero, Kipsitet and Kisian areas to natural pyrethrum formulation, unsynergised pyrethrum, permethrin and deltamethrin

4.8.1 Susceptibility of *An. gambiae* ex-Ahero to the insecticides

Results on susceptibility of *Anopheles gambiae* ex-Ahero area, to the various insecticides is shown in Table 14. It is observed that the natural pyrethrum formulation at the diagnostic dose of 1% had a high early knockdown of 99% at 15min exposure time and this moved up to 100% from 30 min post-exposure period. At 24 hours there was 97% mortality of the mosquitoes and this translated into a resistance index of 3.2%, which was much below the WHO criteria of 30% (WHO, 1998). The non-formulated pyrethrum 1% (technical grade) achieved 95% early knockdown at 15 minutes, which then increased to 97% at 30 minutes. However, the product achieved only 70% mortality at 24 hours and this resulted into a resistance index of 30% proportion, which is classified as resistant as per the standard criteria (WHO, 1998).

On the other hand, deltamethrin (0.5%) achieved an early knockdown of 8% at 15 minutes exposure time but this increased to 77% at 30 minutes and 88% after 60 minutes. The product realized 93% mortality giving a 6.5% resistance index. Permethrin (0.75%) also showed low early knockdown of 13% at 15 minutes exposure time which increased to only 57% at 30 minutes and 88% at 24 hours. The product achieved 80% mortality at 24 hours giving a 14% resistant proportion. The untreated control did not exert any mortality or knockdown.

Table 14: Susceptibility and resistance levels of *Anopheles gambiae* s.l. ex-Ahero to natural pyrethrum formulation, permethrin and deltamethrin

Insecticide	Number assayed (N)	KD(%)-15min ± SE	KD(%)-30min ± SE	KD(%)-60min ± SE	Mortality (%) ± SE	% Resistant (100-% mortality)
Natural pyrethrum formulation	100	99 ± 1.0	100 ±0.0	100. ± 0.0	96.8 ± 1.32	3.2
Deltamethrin 0.5%	100	8.0 ± 3.0	77 ± 6	88.8 ± 1.8	93.15 ± 2.3	6.5
Permethrin 0.75%	100	13.0 ± 2.0	57 ± 6	88.5 ± 2.3	86.1 ± 6.7	13.9
Unformulated pyrethrum (Technical) 1%	100	95 ± 3.0	97 ± 1	92.7 ± 0.1	70 ±5.9	30
Untreated-control	100	0.00	0.00	0.00	0.00	0.00

*Data in the table are means (± standard error). Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while KD (%) 30min, KD (%) 60min, shows the proportion of mosquitoes that were knocked down at 30 and 60 minutes respectively while % mortality refers to mosquitoes killed after 24 hours after exposure to the various insecticides. % resistant refers to the difference between 100% kill and the realized mortality.

4.8.2 Susceptibility of *An. gambiae* s.s. Kisumu strain (ex-KEMRI) to the insecticides

Results on susceptibility of laboratory bred *An. gambiae* S.S. ex-KEMRI, used as the reference control, to the diagnostic doses of the various insecticides are shown in Table 15. It is observed from the results that natural–pyrethrum formulation at the diagnostic dose of 1% had a high early knockdown of 96% at 15min exposure time and this moved up to 100% from 30 minutes exposure period. At 24 hours, there was 97.5% mortality of the mosquitoes and this translated into a resistance index of 2.5%, which was much below the WHO criteria of 30% index. The non-synergized pyrethrums 1% (technical grade) achieved only a 48% early knockdown at 15 minutes which increased to 100% at 30 minutes. However, the product also achieved high mortality of 97.5 % mortality at 24 hours and this resulted into a resistance index of 2.5% which is classified as no-resistance as per the WHO criteria (WHO, 1998).

Deltamethrin (0.5%) achieved a low early knockdown of 29% at 15 minutes exposure time but this increased to 93% at 30 minutes and 98% after 60 minutes. The product realized 100% mortality after 24 hours. Permethrin 0.75% also showed a low early knockdown of 3% at 15 minutes exposure time, which increased to 18% at 30 minutes and 65% at 24 hours. The product achieved 72.5% mortality at 27.5% resistant proportion. The untreated control did not exert any mortality or knockdown.

Table 15: Susceptibility and resistance of *Anopheles gambiae* s.s. ex-KEMRI to natural pyrethrum formulation, permethrin and deltamethrin

Insecticide	**Number assayed (N)**	**KD(%)-15min ± SE**	**KD(%)-30min ± SE**	**KD(%)-60min ± SE**	**Mortality (%) ± SE**	**% Resistant (100-%mortality)**
Natural pyrethrum formulation	100	96 ± 2.0	100 ± 0.0	98.75 ± 1.1	98.5 ± 1.25	2.5
Deltamethrin 0.5%	100	29 ± 5	93 ± 6	98.75 ± 1.25	100 ± 00	0.00
Permethrin 0.75%	100	3 ± 1.0	18 ± 3.0	65 ± 5.6	72.5 ± 2.5	27.5
Unsynergized pyrethrum (Technical) 1%	100	48 ± 25	100 ± 0.0	100 ± 0.0	97.5 ± 0.25	2.5
Untreated-control	100	0.00	0.00	0.00	0.00	0.00

*Data in the table are means (± standard error). Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while KD (%)-30min, KD (%)- 60min, shows the proportion of mosquitoes that were knocked down at 30 and 60 minutes.

4.8.3. Susceptibility of wild *An. gambiae* s.l. ex-KISIAN to the insecticides

Results on susceptibility of wild *An. gambiae* s.l. ex-Kisian to the diagnostic doses of the various insecticides are shown in Table 16. It was observed that natural pyrethrum formulation at the diagnostic dose of 1% achieved high knockdown levels of 100% from 15-60 minutes post-treatment and 100% mortality at 24 hours, translating into a 0-resistance index. The same level of response was also observed with the unformulated pyrethrins 1% (Technical Grade).

Deltamethrin (0.5%) achieved a low early knockdown of 77.5% at 15 minutes exposure time but this increased to 95% at 30 minutes and 96% after 60 minutes. The product realized 100% mortality after 24 hours showing neither resistance nor tolerance by mosquitoes. Permethrin (0.75%) showed a low early knockdown of 2% at 15 minutes post-exposure time which increased to 40% at 30 minutes and 88% at 24 hours. The product achieved 93% mortality with only 7% resistance index. The untreated control did not exert any mortality or knockdown.

Table 16: Susceptibility and resistance of *Anopheles gambiae* s.l. ex-KISIAN to pyrethrins, pyrethrins and deltamethrin

Insecticide	Number assayed (N)	KD(%) - 15 min ± SE	KD(%)- 30min ± SE	KD(%)- 60min ± SE	Mortality (%) ± SE	% Resistant (100-%mortality)
Natural pyrethrum-formulation	100	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	0.00
Deltamethrin 0.5%	100	77.5 ± 1.5	95 ± 2.0	96.3 ± 1.3	100 ± 00	0.00
Pyrethrins 0.75%	100	2.0 ± 2.0	40 ± 8.0	88 ± 4	93.0 ± 3	7.0
Unsynergized pyrethrum (Technical) 1%	100	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	0.00
Untreated-control	100	0.00	0.00	0.00	0.00	0.00

*Data in the table are means (± standard error) unless otherwise stated. Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while KD (%) 15min, KD (%)-30min, KD (%)- 60min, shows the proportion of mosquitoes that were knocked down at 30 and 60 minutes, respectively, while % mortality refers to mosquitoes killed after 24 hours after exposure.

4.8.4 Susceptibility of *Anopheles gambiae* s.l. ex-KIPSITET to the insecticides

Results on susceptibility of wild *An. gambiae* s.l. ex-Kipsitet to the various insecticides are shown in Table 17. Natural pyrethrum formulation at the diagnostic dose of 1% achieved high early knockdown levels of 100% from 15-60 minutes and 100% mortality at 24 hours post treatment giving a zero resistance index. The same level of response was also observed with the unformulated pyrethrum 1% (technical grade).

Deltamethrin (0.5%) achieved a low early knockdown of 6% at 15 minutes exposure time but this increased to 35% at 30 minutes and 100% after 60 minutes. The product realized 97.5% mortality after 24 hours showing a resistance index of 2.5%. Permethrin (0.75%) also showed a low early knockdown of only 11% at 15 minutes post-exposure time which increased abruptly to 87% at 30 minutes and 97% at 24 hours. The product achieved 96.3% mortality, giving a resistance index of only 3.7%. However, the untreated control did not exert any mortality or knockdown.

Table 17: **Susceptibility and resistance of wild *Anopheles gambiae* s.l. ex-KIPSITET area to pyrethrins, permethrin and deltamethrin**

Insecticide	Number assayed (N)	KD(%) -15 min ± SE	KD(%)-30min ± SE	KD(%)-60min ± SE	Mortality (%) ± SE	% Resistant (100-%mortality)
Natural pyrethrum-formulation	100	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	0.00
Deltamethrin 0.5%	100	6.255 ± 2.4	35 ± 3.5	100 ± 0.0	97.5 ±1.4	2.5
Permethrin 0.75%	100	11.25 ±1.25	87.5± 1.4	97.5 ±1.4	96.3 ±1.4	3.7
Pyrethrum (Technical) 1%	100	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	0.00
Untreated-control	100	0.00	0.00	0.00	0.00	0.00

*Data in the table are means (± standard error). Insecticide refers to the tested product at the diagnostic concentrations. Data in columns represented by (N) shows the number of mosquitoes tested while KD (%)-15min, KD (%)- 30min, KD (%)- 60min, shows the proportion of mosquitoes that were knocked down at 15, 30 and 60 minutes, respectively, while % mortality refers to mosquitoes killed after 24 hours after exposure.

4.9 Effect of natural pyrethrum formulation impregnated nets on feeding inhibition and repellency of mosquitoes in tunnel tests

The effect of the nets treated with natural pyrethrum formulation on the mosquito feeding inhibition and irritancy of *Anopheles gambiae* s.s. is shown in Table 18. The results show that dose of pyrethrum formulation impregnated on a net significantly (P=0.013) influenced the number of mosquitoes prevented from accessing the rabbit host through the simulated holes. For instance, there were 76% mosquitoes prevented from accessing the host in the nets treated with natural pyrethrum formulation at 500mg/m^2 as compared to 68% in the nets treated at 250 mg/m^2 and 52% in the untreated net.

It was further observed that even for the mosquitoes that accessed the rabbit host, the treatment dose significantly (P=0.003) affected the feeding success with only 11% feeding from the nets treated at 500 mg/m^2, as compared to 27% in the nets treated at a lower dose of 250 mg/m^2 and 86% in the untreated nets (Table 18). Feeding inhibition was, thus, calculated to be 93% for the nets treated with 500 mg/m^2 and 80% for nets treated at 250 mg/m^2 pyrethrins in the natural pyrethrum formulation.

Table 18: **Effect of nets impregnated with natural pyrethrum formulation on feeding inhibition of *Anopheles gambiae* s.s. in tunnel tests**

Dose (mg/m^2)	%Passage inhibition of mosquitoes ± SE	No of mosquitoes fed	% Mosquitoes fed ± SE	%Blood feeding inhibition
0	52.0 ±4.69^b (7.3)	20.3 ±1.5^a	86.4 ± 1.14^a (9.3)	0
250	68.0 ± 4.97 ^{a} (8.3)	4.0 ± 1.5^b	27.7 ± 12.08^b (4.8)	80.3
500	76.0 ± 3.56 ^{a} (8.8)	1.5 ± 0.65^c	11.08 ± 4.06^b (3.2)	92.6
P value	0.013	0.003	0.003	
LSDα = 0.05	14.22 (0.91)	4.05	23.6 (2.95)	

*Data in the table are means (± standard error). Dose (mg/m^2) refers to the amount of pyrethrins in natural pyrethrum-formulation impregnated on nets expressed in mg/m^2. Data in columns represented by % passage inhibition of mosquitoes, No. of mosquitoes fed, % of mosquitoes fed shows the proportion of mosquitoes that were inhibited from accessing the host, numbers that fed and proportion of attracted that fed on host respectively, following exposure to pyrethrins-treated nets through tunnel method % feeding inhibition refers to reduction in number of blood fed mosquitoes in the treated nets compared to the untreated control i.e (NC-NT)/NC x 100; where NC and NT refers to the number of blood-fed mosquitoes in untreated and treated tunnels, respectively. Figures in brackets represent % values transformed into logarithms. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level (α) of 0.05 based on transformed values for percentages and absolute values for numbers. P-values represent the calculated statistical probability levels.

4.10 Behavioural and insecticidal effects of natural pyrethrum formulation treated nets on malaria vectors under field situation

4.10.1 Baseline proportions of malaria vectors collected in the Ahero study houses

Figure 10 present results on population structure of the malaria vectors collected in 24 study houses in Ahero using the PSC for 7 days. The results show that the two main malaria vectors in the area were *An. gambiae* s.l. and *An. funestus*. During the period, a total of 1152 malaria vectors were collected and of these, 84% were *An. gambiae* s.l. while 15.3% were *An. funestus*. A total of 15.3% of the *A. gambiae* s.l were fed and 23% gravid while 58% of *An.* funestus were fed and 23% gravid (Figure 9). Results further showed that of the *An. gambiae* s.l. samples subjected to PCR 90% was *An. arabiensis* while only 10% was *An. gambiae* s.s.

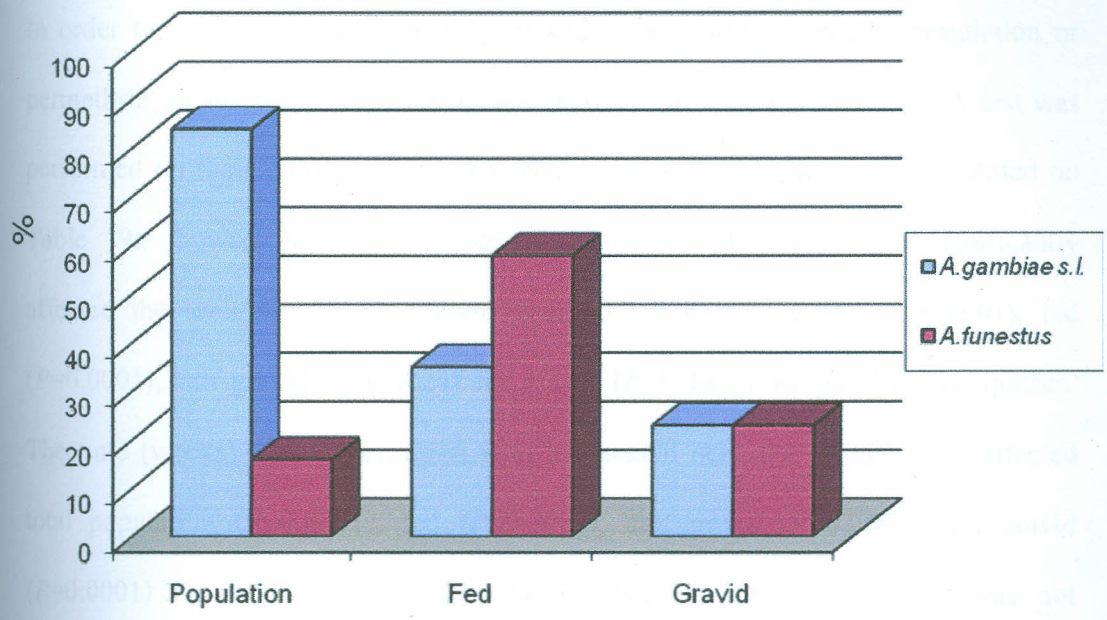

Figure 10: Proportions of malaria vectors and feeding condition before intervention with ITNs.

4.10.2 Influence of nets treated with natural pyrethrum formulation , permethrin or untreated on indoor resting density and feeding behaviour of *Anopheles* mosquitoes under field situation

In order to test the influence of nets treated with natural pyrthrum formulation or permethrin on *Anopheles* mosquitoes under domestic conditions, ANOVA test was performed on their indoor resting and feeding behaviour. The results presented on Table 19a showed that the presence of the insecticide-treated nets significantly affected the total number of indoor resting (P=0.0001), unfed (P=0.0001), fed (P=0.0001), half-gravid (P=0.0224) and gravid (P=0.0002) *An. gambiae* mosquitoes. The time (weeks) after intervention with the treated nets also significantly affected total population (P=0.0001), fed (P=0.0001), half-gravid (P=0.0001) and gravid (P=0.0001) mosquitoes. However, the number of unfed mosquitoes was not significantly (P=0.0876) affected by time of intervention.

There was a significant (P=0.0001) interaction between time after intervention and treatment in influencing total population of mosquitoes recovered in houses (P=0.0001), but interaction did not significantly (P=0.5818) affect the half-gravid *Anopheles gambiae* s.l. mosquitoes (Table 19a).

Table 19a: **ANOVA, probability (P) values on effect of treated nets and time after treatment on indoor resting population and feeding behaviour of *An. gambiae* s.l. In Ahero area**

Source of variation	Total population	Unfed mosquitoes	Fed mosquitoes	Half gravid	Gravid
Treatment	0.0001**	0.0001**	0.0001**	0.0224*	0.0002**
Time	0.0001**	0.0876 ^{NS}	0.0001**	0.0001**	0.0001**
Treatment x time	0.0001**	0.5810 ^{NS}	0.0001**	0.5952	0.0029**

*Data in the table are ANOVA probability values showing influence of treated nets (treatment), time after treatment and interaction of treatment and time on population and feeding behaviour of culicine mosquitoes. Levels of significance are indicated as *** shows high significance at 99.99% level, ** shows siginificance at 99.98, and * depicts not significant at 99. 95% shows significance at while ^{NS}.

Further ANOVA tests (Table 19b) on response of *An. funestus* also showed siginificant influence of treated nets on indoor resting mosquitoes (P=0.0001), fed (P=0.0001), half gravid (P=0.005), gravid(P=0.0005) whith no significant influence (P=0.194) on unfed *An. funestus* mosquitoes. The weeks after intervention (WAI) also significantly affected total population (P=0.0001), fed mosquitoes (P=0.0001) but did not significanly affect the half-gravid (P=0.0587) and gravid (P=0.0587) *An. funestus* mosquitoes.

Table 19b: ANOVA, probability (*P*) values on effect of treated nets and time after treatment on population and feeding behaviour of *An. funestus* in Ahero area

Source of Variation	Total population	Unfed mosquitoes	Fed mosquitoes	Half gravid	Gravid
Treatment	0.0001**	0.194^{NS}	0.0001**	0.0050.**	0.0005.**
Time	0.0001**	0.1961 ^{NS}	0.0001**	0.0587*	0.0587 ^{NS}
Trt X Time	0.0001**	0.1370 ^{NS}	0.0001**	0.00820*	0.0043**

*Data are ANOVA probability values showing influence of treated nets (treatment), time after treatment and interaction of treatment and time on population and feeding behaviour of culicine mosquitoes. Levels of significance are indicated as *** shows high significance at 99.99% level, ** shows siginificance at 99.98, and * depicts not significant at 99 .95% shows significance at while ^{NS}.

In general, there was significantly (P=0.0001) higher numbers of *An. gambiae* mosquitoes collected in houses which had untreated or permethrin treated nets than in houses where there were nets treated with natural pyrethrum-formulation (Table 20). The number of unfed mosquitoes were not significantly (P=0.05) different in houses with natural pyrethrum-formulation and permethrin treated nets. There was significantly (P=0.0001) lower feeding rate of mosquitoes in houses with natural pyrethrum-formulation treated nets than houses with permethrin treated or untreated nets. For instance, an average of 5 mosquitoes per house were fed in houses with pyrethrins treated nets as compared to 10 fed mosquitoes per house in houses with permethrin treated nets and 9 fed mosquitoes in houses with untreated nets. There was also significantly (P=0.0002) higher number of gravid mosquitoes in houses with untreated nets than in the houses with treated nets.

Table 20: Effect of treated nets on indoor resting population and feeding behaviour of *An. gambiae* s.l. mosquitoes in Ahero area

Treatment	Total population	Unfed mosquitoes	Fed mosquitoes	Half gravid	Gravid
Pyrethrum net	16.113^{c} (3.575)	0.920^{b} (1.277)	5.670^{b} (2.250)	0.325^{b} (1.116)	3.025^{b} (1.751)
Permethrin net	17.825^{b} (3.997)	0.725^{b} (1.261)	10.425^{a} (3.054)	0.613^{ab} (1.194)	2.625^{b} (1.751)
Untreated net	23.228 (4.695)^{a}	1.835^{a} (1.583)	8.924^{a} (2.966)	0.8354^{a} (1.274)	4.468^{a} (2.161
LSD(0.05)	(0.4071)	(0.149)	(0.283)	(0.112)	(0.218)
P-Value	0.0001	0.0001	0.0001	0.0224	0.0002

*Data in the table are means. Treatment refers to insecticide used in the bed net, total population refers to numbers of mosquitoes recovered indoors by PSC, unfed, fed. Half gravid and gravid refers to the abdominal conditions of the mosquitoes collected indoors. Figures in brackets represent values transformed into logarithms. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. P-values represent the calculated statistical probability levels.

With respect to *An. funestus,* results in Table 21 shows that there were significanly (P=0.0001) higher numbers collected in houses with nets treated with natural pyrethrum-formulation and untreated nets than in houses with permethrin treated nets. However, there was siginificantly (P=0.0001) lower numbers of mosquitoes fed in houses with nets treated with pyrethrum-formulation or permethrin than in the houses with untreated nets.

Table 21: Effect of treated nets on population and feeding behaviour of *An. funestus*

Treatment	Total population	Unfed mosquitoes	Fed mosquitoes	Half gravid	Gravid
Pyrethrum net	4.85^{b} (2.14)	0.47^{a} (1.17)	1.33^{c} (1.35)	0.063 (1.026)	0.594^{b} (1.204)
Permethrin net	0.81^{a} (1.24)	0.15^{b} (1.05)	0.23^{c} (1.075)	0.0875 (1.034)	0.063^{c} (1.025)
Untreated net	4.96^{b} (2.16)	0.34^{a} (1.13)	2.29^{a} (1.60)	0.316^{a} (1.12)	1.076^{a} (1.354)
LSD(0.05)	(0.213)	(0.08))	(0.169)	(0.051)	(0.096)
P-Value	0.0001	0.0298	0.0001	0.0005	0.0001

*Data in the table are means. Treatment refers to insecticide used in the bed net. Total population refers to numbers of mosquitoes recovered indoors by PSC, unfed, fed. Half-gravid and gravid refers to the abdominal conditions of the mosquitoes collected indoors. Figures in brackets represent values transformed into logarithms. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. P-values represent the calculated statistical probability levels.

Table 22 shows the effect of days after intervention with nets treated with natural pyrethrum formulation on indoor resting population and abdominal status of *An. gambiae* s.l. and *An. funestus*. Results show that there was significantly (P=0.0001) higher population of *An. gambiae* s.l. and *An. funestus* recovered in the experimental houses before intervention (WAI=0) than all the other weeks (WAI=1 – 16) after intervention with the nets treated with natural pyrethrum formulation. This trend was similar on gravid *An. gambiae* s.l. but with no significant (P=0.05) effect on *An. funestus*. Intervention with natural pyrethrum formulation treated nets significantly (P=0.0001) reduced the feeding of *An. gambiae* s.l. only at 1 WAI as compared to before intervention but with no significant (P=0.05) feeding effect on *An. funestus*.

Table 22: Effect of days after intervention with nets treated with natural pyrethrum formulation on indoor resting population and abdominal condition of *An. gambiae* and *An. funestus* malaria vectors

	Total Population		Unfed		Fed		Half Gravid		Gravid	
WAI	***A. gambiae***	***A. funestus***	***A .gambiae***	***A. funestus***	***A. gambiae***	***A.funestus***	***A. gambiae***	***A. funestus***	***A. gambiae***	***A. funestus***
0	65.88^{a} (7.78)	15.29^{a} (3.73)	1.13^{a} (1.39)	0.50^{a} (1.15)	9.25^{c} (4.39)	0.75^{a} (2.27)	1.13^{a} (1.3)	0.00^{a} (1.00)	1.12^{a} (3.53)	2.00^{a} (1.56)
1	6.13^{de} (2.51)	0.50^{d} (1.20)	0.38^{a} (1.14)	0.38^{a} (1.14)	3.13^{d} (1.72)	0.13^{d} (0.00)	0.13^{a} (1.05)	0.00^{a} (1.00)	0.63^{cb} (1.26)	0.00^{d} (1.00)
2	1.00^{e} (1.35)	9.50^{ab} (2.98)	0.38^{a} (1.14)	0.03^{a} (1.05)	9.75^{c} (1.00)	0.13^{ab} (1.75)	0.00^{a} (1.00)	0.25^{a} (1.10)	0.25^{c} (1.10)	0.88^{abc} (1.32)
4	13.88^{bcd} (3.68)	2.13^{cd} (1.63)	0.13^{a} (1.05)	0.50^{a} (1.20)	10.13^{bc} (2.11)	1.00^{c} (1.81)	0.75^{a} (1.27)	0.00^{a} (1.00)	2.75^{b} (1.84)	0.13^{cd} (1.05)
6	17.50^{b} (4.20)	3.50^{bcd} (2.05)	0.88^{a} (1.34)	0.00^{a} (1.00)	11.30^{abc} (2.86)	0.13^{c} (1.00)	0.25^{a} (1.10)	0.00^{a} (1.00)	1.88^{bc} (1.63)	0.38^{bcd} (1.14)
8	9.25^{bcd} (3.08)	2.12^{cd} (1.64)	0.50^{a} (1.15)	0.00^{a} (1.00)	16.80^{ab} (1.87)	0.00^{c} (1.00)	0.00^{a} (1.00)	0.00^{a} (1.00)	2.00^{bc} (1.62)	0.00^{d} (1.00)
10	9.13^{cd} (2.81)	5.63^{bcd} (2.32)	1.00^{a} (1.35)	0.00^{a} (1.00)	10.00^{c} (1.73)	0.00^{ab} (1.00)	0.38^{a} 1.16)	0.13^{a} (1.05	1.38^{bc} (1.46)	0.63^{bcd} (1.25)
12	9.50^{bcd} (3.03)	3.63^{bcd} (1.99)	1.13^{a} (1.41)	0.00^{a} (1.00)	16.75^{a} (2.17)	0.00^{bc} (1.00)	0.25^{a} (1.10)	0.13^{a} (1.05)	2.50^{bc} (1.68)	0.13^{cd} (1.05)
14	12.75^{bcd} (3.43)	1.88^{cd} (1.63)	1.38^{a} (1.47)	0.00^{a} (1.00)	9.75^{bc} (2.21)	0.00^{c} (1.00)	0.13a (1.05)	0.00^{a} 1.00)	1.88^{bc} (1.59)	$0.75^{a\,bcd}$ (1.27)
16	16.13^{bc} (3.89)	5.63^{bc} (2.45)	0.38^{a} (1.16)	0.00^{a} (1.00)	7.50^{c} (2.44)	0.13^{bc} (1.27)	0.25^{a} (1.09)	0.13^{a} (1.05	3.13^{b} (1.83)	1.25^{ab} (1.44)
LSD, α=0.05	1.287	0.674	0.472	0.252	0.896	0.535	0.354	0.16	0.688	0.303
P-value	0.0001	0.0001	0.5810	0.1370	0.0001	0.0001	0.5952	0.00820	0.0029	0.0043

*Data are means unless otherwise stated. WAI refers to weeks after intervention with treated bednet. Total population refers to number of mosquito species recovered indoors by PSC. Unfed, fed, half-gravid and gravid refers to the abdominal conditions of the mosquitoes collected indoors. Figures in brackets represent values transformed into logarithms for ANOVA tests. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic.

Table 23 shows that there was significantly (P=0.0001) higher population of *An. gambiae* recovered in the experimental houses before intervention (WAI=0) with permethrin treated nets as compeared to WAI 1 – 4 only but with no sigificant (P=0.05) reduction observed from WAI 6-12. Significant (P=0.0001) reduction in *An. funestus* population was only observed at 1 WAI. Feeding was also significantly (P=0.0001) affected from 1 WAI in *An. gambiae* as well as gravid mosquitoes (P=0.0029) as shown in Table 22. With *An. funestus,* there was an initial sinificantly higher numbers of gravid mosquities before intervention (P=0.0043) than numbers at 1-2WAI, however from 4WAI there was no significant effect (P=0.05).

Table 23: Effect of days after intervention with permethrin-treated nets on indoor resting population and abdominal condition of *An. gambiae* and *An. funestus* malaria vectors in Ahero area

	Total Population		Unfed		Fed		Half Gravid		Gravid	
WAI	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*
0	26.50^{a} (5.08)	2.63^{a} (1.78)	1.13^{a} (1.39)	0.50^{a} (1.15)	9.25^{b} (2.92)	0.75^{b} (1.27)	2.50^{a} (1.74)	0.63^{a} (1.24)	9.38^{a} (3.11)	0.38^{a} (1.14)
1	9.50^{d} (2.76)	1.88^{d} (1.54)	1.38^{a} (1.14)	0.38^{a} (1.14)	3.13^{c} (1.85)	0.13^{a} (1.05)	1.25^{a} (1.29)	0.13^{a} (1.05)	1.50^{bc} (1.49)	0.25^{b} (1.09)
2	14.0^{bcd} (3.59)	0.38^{a} (1.14)	0.38^{a} (1.14)	0.13^{a} (1.05)	9.75^{b} (2.92)	0.13^{a} (1.05)	0.25^{a} (1.10)	0.13^{a} (1.05)	1.00^{b} (1.38)	0.00^{b} (1.00)
4	16.62^{bc} (3.93)	2.63^{a} (1.73)	0.13^{a} (1.05)	0.50^{a} (1.20)	10.13^{b} (3.09)	1.00^{a} (1.28)	0.5^{a} (1.19)	0.00^{a} (1.00)	2.88^{bc} (1.85)	0.00^{a} (1.00)
6	19.63^{ab} (4.38)	0.13^{a} (1.05)	0.88^{a} (1.34)	0.00^{a} (1.00)	11.25^{ab} (3.37)	0.13^{a} (1.05)	0.63^{a} (1.24)	0.00^{a} (1.00)	2.13^{bc} (1.71)	0.00^{a} (1.00)
8	22.25^{ab} (4.51)	0.25^{a} (1.09)	0.50^{a} (1.15)	0.00^{a} (1.00)	16.75^{a} (3.80)	0.00^{a} (1.00)	0.38^{a} (1.14)	0.00^{a} (1.00)	1.50^{bc} (1.51)	0.00^{a} (1.00)
10	16.13^{bc} (3.86)	0.00^{a} (1.00)	1.00^{a} (1.35)	0.00^{a} (1.00)	10.00^{b} (3.01)	0.00^{a} (1.00)	0.00^{a} (1.00)	0.00^{a} (1.00)	1.63^{b} (1.58)	0.00^{a} (1.00)
12	23.88^{ab} (4.53)	0.00^{a} (1.00)	1.13^{a} (1.41)	0.00^{a} (1.00)	16.75^{a} (3.89)	0.00^{a} (1.00)	0.00^{a} (1.00)	0.00^{a} (1.00)	2.50^{b} (1.78)	0.00^{a} (1.00)
14	19.25^{ab} (4.29)	0.13^{a} (1.05)	1.38^{a} (1.14)	0.00^{a} (1.00)	9.75^{b} (3.04)	0.00^{a} (1.00)	0.50^{a} (1.17)	0.00^{a} (1.00)	2.75^{cd} (1.79)	0.13^{a} (1.05)
16	19.50^{cd} (3.10)	0.13^{a} (1.05)	0.50^{a} (1.16)	0.00^{a} (1.00)	7.50^{b} (2.65)	0.13^{a} (1.05)	0.13^{a} (1.05)	0.00^{93} (1.00)	1.00^{d} (1.33)	0.00^{a} (1.00)
LSD, α=0.05	1.287	0.674	0.472	0.252	0.896	0.535	0.354	0.163	0.688	0.303
P-Value	0.0001	0.0001	0.5810	0.1370	0.0001	0.0001	0.5952	0.00820	0.0029	0.0043

*Data are means unless otherwise stated. WAI refers to weeks after intervention with treated bednet, total population refers to numbers of mosquito species recovered indoors by PSC. unfed, fed. Half gravid and gravid refers to the abdominal conditions of the mosquitoes collected indoors. Figures in brackets represent values transformed into logarithms. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic probability levels.

Table 24 shows that there was significantly (P=0.0001) higher population of *An. gambiae* s.l. recovered in the experimental houses before intervention (WAI=0) than the mosquito populations recoverd at 1-4 WAI with untreated nets, however, at 6-16 WAI there was no significant (P=0.05) reduction. With *An. funestus*, significantly (P=0.0001) higher population was observed only at 16 WAI. Feeding of *An. gambaie* was also significantly (P=0.0001) reduced by introduction of untreated nets for 1-2 WAI with significant (P=0.0001) effect on *An . funestus.*

Table 24: Effect of days after intervention with untreated nets on indoor resting population and abdominal status of *An. gambiae* and *An. funestus* malaria vectors in Ahero area

	Total Population		Unfed		Fed		Half Gravid		Gravid	
WAI	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*
0	29.63^{a} (5.19)	5.38^{b} (2.48)	3.00^{a} (1.87)	0.63^{a} (1.18)	11.63^{a} (3.37)	2.38^{b} (1.78)	3.13^{a} (1.87)	0.63^{a} (1.25)	4.88^{ab} (2.25)	0.88^{a} (1.32)
1	17.00^{bc} (3.96)	2.13^{b} (1.68)	1.57^{a} (1.44)	0.25^{a} (1.10)	3.29^{c} (1.99)	0.63^{b} (1.21)	0.43^{c} (1.16)	0.00^{c} (1.00)	1.14^{d} (1.35)	0.88^{a} (1.32)
2	15.25^{c} (3.65)	2.86^{b} (1.76)	1.25^{a} (1.46)	0.14^{a} (1.06)	4.87^{bc} (2.21)	2.00^{b} (1.58)	0.63^{c} (1.22)	0.00^{c} (1.00)	1.88^{cd} (1.60)	0.43^{a} (1.14)
4	20.88^{abc} (4.49)	3.13^{b} (1.88)	1.25^{a} (1.42)	0.13^{a} (1.05)	10.25^{a} (3.26)	1.50^{b} (1.48)	0.50^{c} (1.20)	0.25^{abc} (1.10)	5.38^{ab} (2.35)	1.13^{a} (1.39)
6	25.75^{ab} (4.98)	3.00^{b} (1.86)	2.25^{a} (1.75)	0.5^{a} (1.21)	7.25^{abc} (2.77)	0.38^{b} (1.14)	1.50^{b} (1.52)	0.38^{abc} (1.16)	7.75^{a} (2.63)	0.63^{a} (1.23)
8	26.13^{ab} (4.99)	2.88^{b} (1.77)	1.5^{a} (1.49)	0.13^{a} (1.05)	11.63^{a} (3.35)	1.75^{b} (1.39)	0.13^{c} (1.05)	0.13^{bc} (1.05)	5.50^{ab} (2.48)	0.38^{a} (1.16)
10	24.63^{ab} (4.97)	3.88^{b} (2.02)	1.5^{a} (1.49)	0.5^{a} (1.20)	9.88^{a} (3.16)	1.50^{b} (1.45)	0.88^{bc} (1.31)	0.63^{a} (1.24)	3.88^{abc} (2.16)	0.75^{a} (1.28)
12	22.50^{abc} (4.72)	8.88^{b} (2.65)	2.25^{a} (1.76)	0.5^{a} (1.20)	9.63^{ab} (3.00)	4.38^{b} (1.85)	0.25^{c} (1.10)	0.36^{abc} (1.16)	3.63^{bc} (2.02)	1.75^{a} (1.43)
14	22.38^{abc} (4.69)	3.13^{ab} (1.82)	1.38^{a} (1.45)	0.13^{a} (1.05)	9.63^{a} (3.17)	1.75^{b} (1.49)	0.50^{c} (1.18)	0.13^{bc} (1.05)	0.50^{d} (1.18)	0.75^{a} (1.27)
16	27.38^{a} (5.23)	14.13^{a} (3.68)	2.38^{a} (1.68)	0.5^{a} (1.20)	10.50^{a} (3.27)	6.63^{a} (2.60)	0.38^{c} (1.13)	0.63^{ab} (1.18)	0.38^{d} (1.13)	3.13^{b} (1.98)
LSD, α=0.05	1.287	0.674	0.472	0.252	0.894.	0.535	0.354	0.163	0.688	0.303.
P-value	0.0001	0.0001	0.5810	0.1370	0.0001	0.0001	0.5952	0.0082	0.0029	0.0043

*Data are means unless otherwise stated. WAI refers to weeks after intervention with treated bed net, total population refers to numbers of mosquito species recovered indoors by PSC. unfed. fed. Half-gravid and gravid refers to the abdominal conditions of the mosquitoes collected indoors. Figures in brackets represent values transformed into logarithms. Means in same column with same superscript letter are not significantly different according to least significance difference (LSD) test at probability level of 0.05 based on transformed values. *P*-values represent the calculated statistic probability levels.

4.10.3 Influence of bednets treated with natural pyrethrum formulation, permethrin and untreated nets on proportional reduction of indoor resting density and feeding inhibition of *An. gambiae* s.l. and *An. funestus* under field situation

Figure 11 shows the effect of bednets treated with natural pyrethrum formulation on proportional reduction of indoor resting and feeding of *An. gambiae* and *An. funestus* relative to the baseline population observed before intervention. The results show a drastic reduction of 83.4% on indoor resting mosquitoes at one week after intervention (1 WAI) and 77.5% at 2 WAI. This proportion was continuously sustained up to 65% at 16 WAI in *An. gambiae*, (Figure 11). A similar trend was observed for *An. funestus* which showed 86.6% reduction in indoor resting population at 1 WAI, 83.7% at 2 WAI and 47% at 16 WAI.

Results in Figure 11 further shows consistent reduction in the proportion of fed *An. gambiae* collected in houses with bed nets treated with natural pyrethrum-formulation. For instance, there was a feeding reduction of 77% at 1 WAI, 70% at 2 WAI and 60% at 16 WAI. There was however, lower feeding reduction in An. *funestus* resulting in 50% at 1WAI, 50% at 2 WAI, and 48% at 16 WAI.

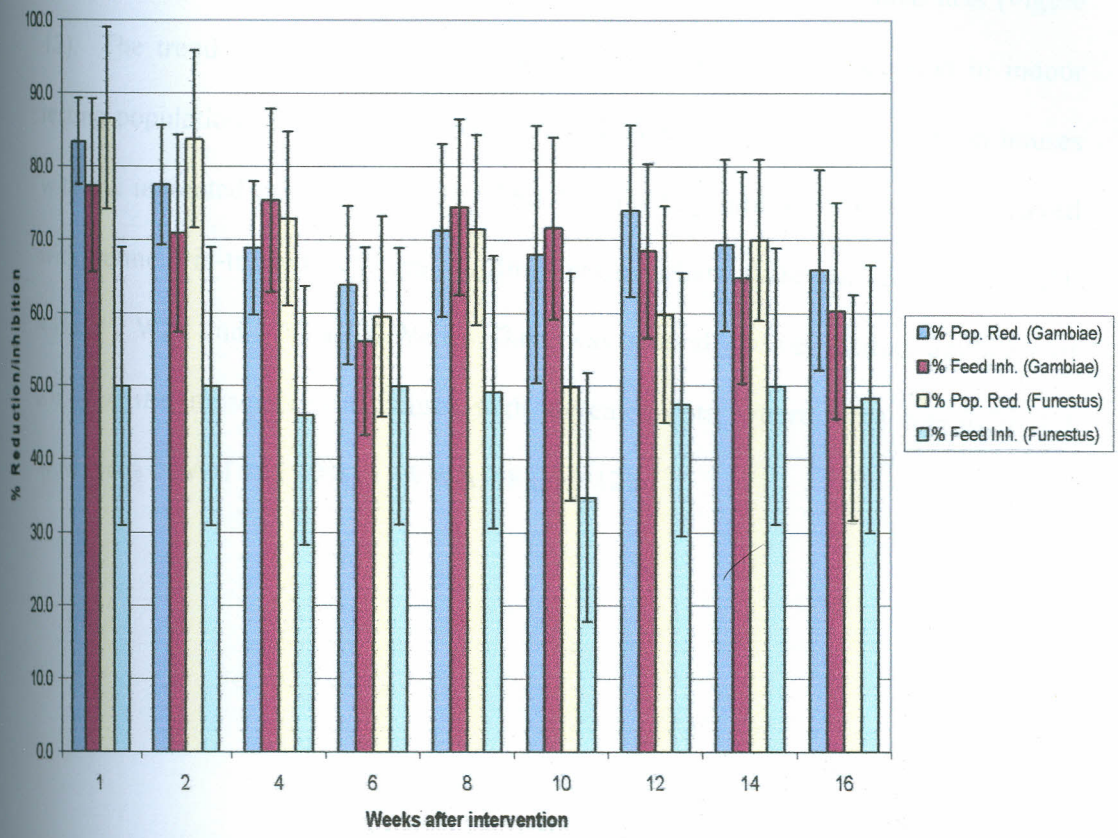

Figure 11: Effect of bednets treated with natural pyrethrum formulation on proportional reduction of indoor resting and feeding in *An. gambiae* s.l. and *An. funestus*

*Figures are calculated mean % indoor resting population reduction/feeding inhibition calculated as, 100 x Number before treatment (NBT) –Number after treatment (NAT)/NBT. WAI represents weeks after intervention during which observations were taken.

In contrast to the bednets treated with natural pyrethrum formulation, in houses where untreated bednets were present, there was only 47.7% reduction in population at 1 WAI, 52% at 2 WAI and 14% at 16 WAI in the houses with untreated nets (Figure 12). The trend was similar in *An. funestus* where there was reduction in indoor resting population of 60% at 1 WAI, 64% at 2 WAI and 45% at 16 WAI in houses with the untreated nets (Figure 12). This low feeding inhibition was also observed with permethrin-treated nets against *An. funestus* where there was 31% at 1 WAI, 44% at 2 WAI and 37% at 16 WAI. There was generally lower feeding inhibition of *An. funestus* mosquitoes in houses with untreated nets, where there was 67% at 1 WAI, 46% 2 WAI and 20% at 16 WAI (Figure 12).

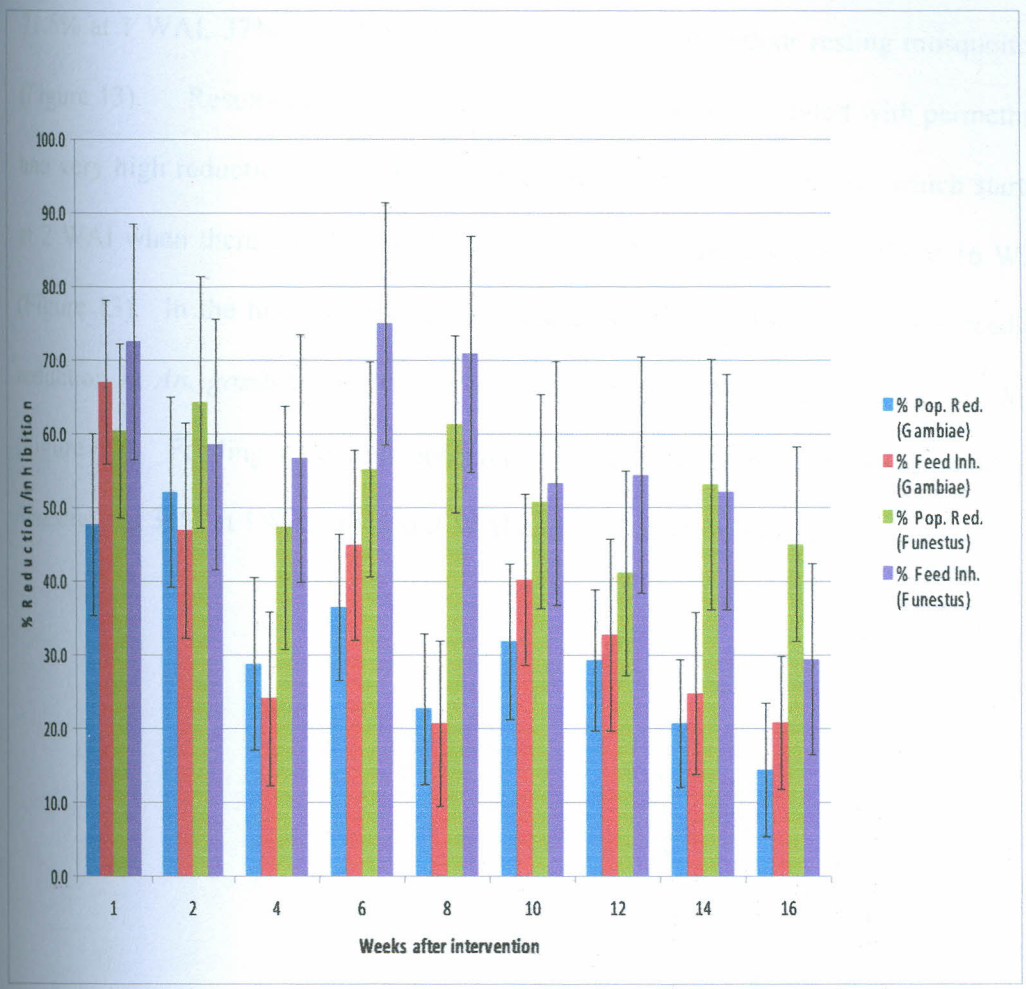

Figure 12: Effect of untreated bednets on proportional reduction of indoor resting and feeding in *An. gambiae* and *An. funestus*.

* Figures are calculated mean % indoor resting population reduction/feeding inhibition calculated as 100xNumber before treatment(NBT)–Number after treatment (NAT)/NBT with bars representing standard errors of the means. WAI represents weeks after intervention during which observations were taken.

In houses where there were bednets treated with permethrin, there was a reduction of 71.5% at 1 WAI, 37% at 2 WAI and 51% at 16 WAI in indoor resting mosquoitoes (Figure 13). Results further show that houses with bednets treated with permethrin had very high reductions on indoor resting populations of *An. funestus* which started at 2 WAI when there was 85% reduction and this was increased to 99% at 16 WAI (Figure 13). In the houses with bednets treated with permethrin, there was feeding reduction in *An. gambiae* of 59% at 1 WAI, 24% at 2 WAI and 41% at 16 WAI (Figure 13). Feeding reduction observed with permethrin-treated nets against *An. funestus* was 31% at 1WAI, 44% at 2 WAI and 37% at 16 WAI (Figure 13).

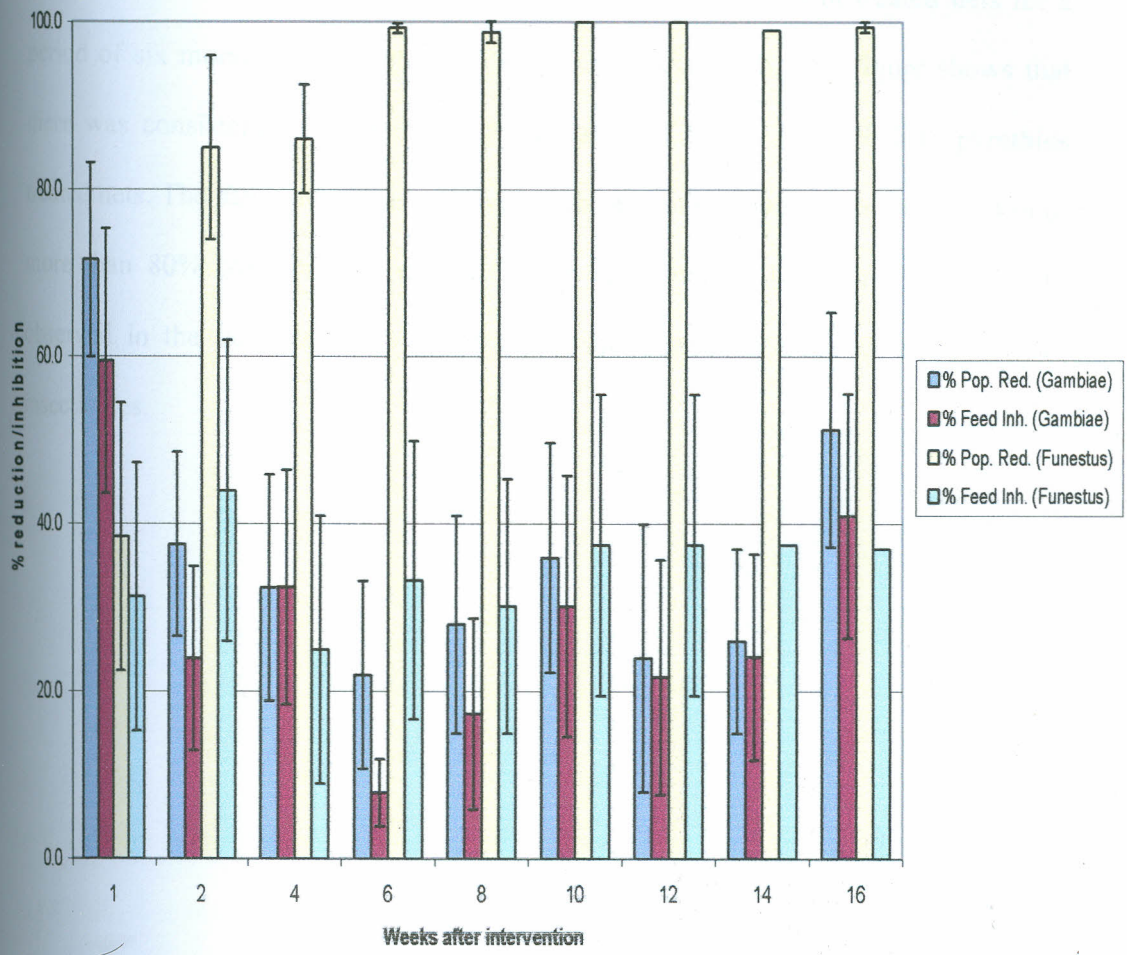

Figure 13: Effect of bednets treated with permethrin on proportional reduction of indoor resting and feeding in *An. gambiae* s.l. and *An. funestus*.

* Figures are calculated mean % indoor resting population reduction/feeding inhibition calculated as, 100 x Number before treatment(NBT)–Number after treatment (NAT) /NBT with bars representing standard errors of the means. WAI represents weeks after intervention during which observations were taken.

4.10.4 Bio-efficacy persistence of pyrethrum-formulation and permethrin treated nets under field situation

Figure 14 shows the bio-persistence of pyrethins and permethrin treated nets for a period of six months under field situation without any wash. The figure shows that there was consistently high percentage mortality of higher than 90% in pyrethins treated nets. The same trend was observed with permethrin, with high levels of kill of more than 80% over the test period. There was however less than 5% mortality observed in the untreated nets underscoring the significance of net treatmet with insecticides.

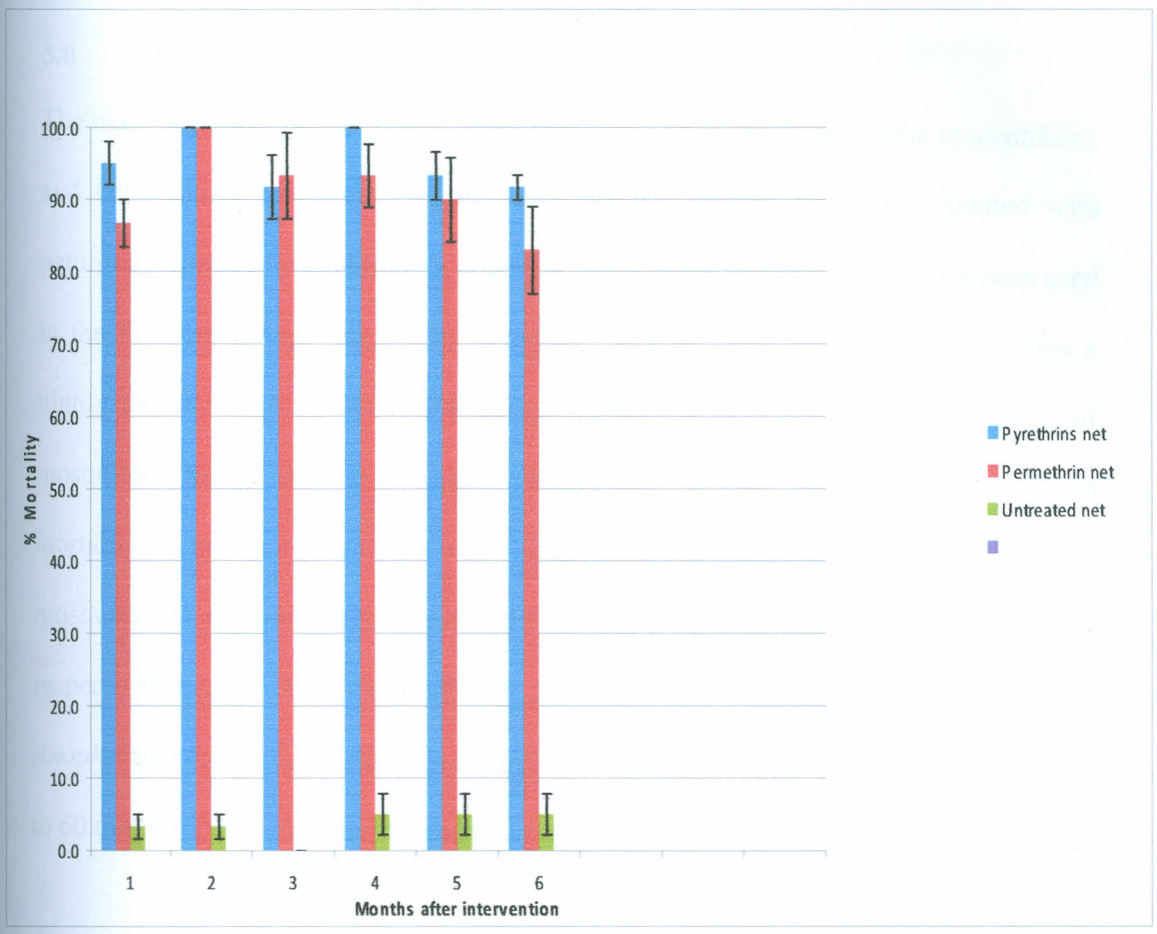

Figure 14: Bio-efficacy persistence of pyrethrum formulation and permethrin-treated nets over six month period under field situation.

CHAPTER FIVE

5.0 DISCUSSION

The main objective of the current study was to evaluate and quantify the susceptibility and behavioural responses of *Anopheles* malaria vectors to bednets treated with natural pyrethrum formulation , a botanical insecticide product that has not been used in the ITN technology despite its potential. Results demonstrated that there was a highly significant (P=0.0001) interaction between dose and time post-exposure of mosquitoes to the natural pyrethrum formulation treated net on knockdown and mortality of mosquitoes. There was however no significant additional effect on mosquito KD at durations longer than 15 minutes post exporsue the treated nets at the respective doses. Thus, bio-efficacy test to assess knockdown (KD) effect on *Anopheles* mosquitoes could be standardized at 15 minutes post-exposure as opposed to 60 minutes currently recommended, in order to save on time and resources.

The results further show that the pyrethrum-formulation is suitable for impregnation of polyester nets at 500mg/m^2 when it achieves WHO specified standard residual bioefficacy (WHO, 1996) upto 6 months on nets, and allows for three washes without compromising bio-efficacy. In the event of wear or tear, the bednets treated with natural pyrethrum-formulation have good deterrence effect while mosquitoes that access the host seem to loose the ability to feed, thus minimizing their vector competence. In addition, the study showed that the natural pyrethrum-formulation was most suitable for treatment of nylon and polyester fabrics with a rather poor performance on cotton nets. This demonstrates the significance of the intrinsic fabric properties and dosage level of the insecticide used for impregnation on the bioefficay of ITNs. The study also reveals that the use of coloured treated nets may be good in

initiating immediate knockdown of mosquitoes on contact. Throughout the study, *An. gambiae* s.l. wild populations were completely susceptible to the natural pyrethrum formulation.

The high susceptibilty of the *An. gambiae* with fixed *kdr* alleles to the natural pyrethrum formulation shows its potential for use to manage *kdr* resistance in mosquitoes. Understanding variations in susceptibilty of *An. gambiae* s.l. populations to different insecticides is key to successful implementation of ITNs in malaria control initiatives. The reduced population and feeding of mosquitoes in the houses with bednets treated with natural pyrethrum formulation further shows its effectiveness possibly through dissuasive effect and reducing the availability of mosquitoes that would effectively bite to transmit disease or cause nuisance.

5.1 Formulation, dose effects, persistence, feeding inhibition and wash resistance of bednets impregnated with a natural pyrethrum formulation against *An. gambiae* s.s.

Formulation involves complex physical and chemical interactions that occur between various components put together. It greatly influences the bio-availability, performance and stability of the active ingredient (Foy and Pritchard, 1996). In the current work, the natural pyrethrum formulation has shown good properties, including emulsification in water allowing for "dip-it-yourself" impregnation of bednets. Pyrethrins are polar in nature and are not easily miscible with water but the added emulsifiers usually have varying units of ethylene oxide to give greater affinity for water (Casida and Quistard, 1995). The good binding property of the formulation with the treated netting fibre may explain the reason for the observed wash-resistance

and prolonged residual bio-efficacy of the pyrethrins-treated net in the current study. This could be because of the fact that the polymer chains in the synthetic nets have same polarity as pyrethrins which are non-volatile and are high-boiling point esters (Casida and Quistard, 1995), properties that enhance the diffusion of pyrethrum into the net. Addition of the synergist in the formulation is of interest, because they act on resitant speceis by inhibition of mixed function oxidase and esterase enzymes (Vulule *et al.*, 1999; Ramoutar *et al.*, 2009). However, in the current study, it has been reveaeld that addition of a synergist and a stabilizer in the natural pyrethrum-formulation had a significant effect on resistant mosquitoes with *kdr* genes. This is considered crucial because of the potential threat that this form of resistance pauses to the success of ITNs (Chouaibou *et al.*, 2008; Yewhalaw *et al.*, 2011).

The long persistence of the formulation on treated nets could possibly be due to the addition of the synergist that have been shown to stabilize some insecticides and hence increasing potential for bio-persistence (www.endura.int). Besides, the addition of an anti-oxidant had the potential to enhance stability of the natural pyrethrum formulation by binding the oxygen molecules thus reducing the oxidative process which is the main degradation route in pyrethrum (Casida and Quistard, 1995). The anti-oxidant may also interfere with mixed-function oxidase enzymes produced by insects in response to presence of an insecticide (www.endura.int) thus increasing the potentilal of this formulation to fight resistance. Stability of the formulation is important since it improves on the cost effectiveness of the treated net. Under conventional situations, a treated net should last for a minimum of six months with an allowance of, at most, two washes before re-treatment (WHO, 1998). Studies have also shown that nets re-treated in less than six months had better impact than

those not re-treated (Marchant *et al.*, 2001). The fact that the natural pyrethrum formulation treated nets could sustain upto three washes makes the net conforms to previously observed practices (Maxwell *et al.*, 2003) where nets were generally washed 2 or 3 times per year.

The study has also shown that washing significantly reduces efficacy of a treated net and this has been observed previously in other studies (Ordonez-Gonzalez *et al.*, 2002; Rafinejad *et al.*, 2008), although with differences in magnitudes depending on the treatment and insecticide formulation. In the current study, it was observed that washing a bednet three times reduced the initial dosage of pyrethrins by 70.6% while in other studies, the same number of washings in traditional manner, for example, using cow-fat soap, reduced initial dosages by about 85% for cypermethrin and 99.8% for pirimiphos methyl, but left no detectable residues for deltamethrin (Rafinejad *et al.*, 2008). The relatively low wash fastness of natural pyrethrum-formulation impregnatred on nets may also be due to its low solubility in water besides the polymer blending. Studies elsewhere have also shown that in some instances, insecticidal effect is not usually appreciated as most users will prefer a clean net at the risk of losing entomological impact (Fielden, 1996), hence the need for strong information, education and communication component in bednet usage.

The observed dose-dependent, reduced feeding of mosquitoes in the holed-treated nets, compared to the untreated nets, does confirm the significance of natural pyrethrum-formulation in treatment of nets. In the current study, a 86% feeding success in the untreated nets was observed. This observation was higher compared to

28% and 11% for nets treated with natural pyrethrum-formulation at 250mg/m^2 and 500mg/m^2. In studies elsewhere, 45-70% of the mosquitoes were able to feed on the host with untreated nets (Corbel *et al.,* 2004). Differences observed in the current versus previous studies may have been due to specific feeding and resting behaviour patterns of different mosquito species that could influence their reaction to different insecticides. It is of interest that despite a large number of mosquitoes accessing the host, only a small percentage was able to feed. The underlying reason for this is unclear, but may be due to some other salient properties of pyrethrins, such as spatial repellent effect that has been previously reported (Casida and Quistard, 1995), probably, coupled with volatile additives in the formulation. It is also thought that pyrethrins have a "jamming" effect where mosquitoes exposed to pyrethrins get confused and stop seeking blood meals (www.bugfreebackyards.com). This jamming phenomenon opens up a new arena for natural pyrethrum use in treated nets, especially where the nets get torn or worn out through various handling and use situations, resulting in the nets still offering substantial protection to the user.

High mortality of more than 90% observed with the recommended dose of 500mg/m^2 is crucial because in most malaria control programmes, ITN coverage is often less than 100% (Mosha *et al.,* 2008), so reduction of vector population remain an important strategy.

5.2 Effect of different fabrics and colour on bio-efficacy of natural pyrethrum formulation impregnated nets against *An. gambiae* s.s. mosquitoes

Appropriate choice of a netting fabric for use in insecticide-treated nets (ITNs) is crucial and is closely related to the compatibility of the fabric with insecticide

formulation (Rozendaal, 1989; WHO, 2006). In this study, it has been demonstrated that pyrethrum formulation works best on nylon, followed by polyester with the worst bio-efficacy observed in the cotton material. The relatively good compatibility in terms of bio-efficacy of the pyrethrum formulation in the synthetic nylon and polyester fabrics is probably due to the fact that the polymer chains in nylon and polyester have same polarity as pyrethrins which are non-volatile and are high boiling point esters (Casida and Quistard, 1995) resulting in no antagonistic activity. However, the better performance of nylon over polyester may be attributable to its thinner yarns that allowed easier availability of insecticide for contact with the mosquito. The observed differences in bio-efficacy of the different insecticide-treated netting fabrics in the current study are consistent with previous findings on pyrethroids-impregnated nets (Vatandoost *et al.*, 2006). In these studies, there was relatively poor bio-efficacy on treated cotton nets against mosquitoes, although the insecticide formulation performed better in polyester fabric used than in nylon.

The high level of knockdown experienced with the treated nylon net at all doses in the current study supports previous studies in which a low dose of 25 mg/m^2 of cyfluthrin and deltamethrin, impregnated on nylon net, resulted in 100% mortality on female mosquitoes that landed on the fabric (Ansari and Razdan, 2000). However, in a different study with various synthetic pyrethroids on netting fabrics (Vatandoost *et al.*, 2006), it was observed that polyester achieved a better bio-efficacy than nylon thus underscoring the importance of the nature of formulation on the bio-performance of an impregnated material The poor performance of the impregnated cotton nets is probably because of the texture of the cotton fabric; being rough, porous and absorbent. This allows uptake of more insecticide than the synthetic materials,

however, a substantial part of the insecticide is not contactable on the surface resulting in the possibility of the mosquito picking up sub-lethal doses (Lines *et al.*, 1987; Rozendaal, 1989), hence the low knockdown and kill. The fact that cotton nets contain starch which dissolves during impregnation can affect the binding and bio-availability of insecticide for contact by the mosquito (Nadanathangam *et al.*, 2006).

The observed significant interaction between insecticide dose and fabrics, pointed out that the performance of a given dose of insecticide was not uniform among the fabrics. This is further confirmed by the regression models that showed higher regression coefficients in the synthetic fabrics of nylon and polyester than in the natural cotton fabric. The KD_{95} and LC_{80} values on knockdown and mortality values show that a much higher dose of insecticide is needed to impregnate cotton nets for these fibers to realize the same effect as polyester and nylon. This has been demonstrated in previous studies in which it was observed that the texture of a net determines the target insecticide concentration necessary for its impregnation (Rafinejad *et al.*, 2008). This suggests that insecticide-impregnated cotton net would be more costly and this can adversely impact on demand of the material, resulting in less usage.

Affordability of a product is normally positively correlated with its acceptance by users. Insecticide-impregnated net that is relatively inexpensive would, therefore, earn consumers preference thus contributing to the success of malaria control effort. It has been observed that in the South-East Asian countries, local availability of cheap mass-produced mosquito nets enhances large-scale use of ITNs, while in Africa and South American countries, the higher prices of locally made mosquito nets hamper

large-scale use (Rozendaal, 1989). Besides, the relatively lower impregnation dosage required for synthetic nylon and polyester fabrics, makes them cheaper, and more preferable than cotton nets (Rozendaal, 1989). The fact that at an equivalent dose, the pyrethrum formulation under evaluation was comparable to the standard permethrin formulation at the recommended concentration of 500 mg/m^2 confirms the suitability of the natural pyrethrum-formulation for use in impregnated nets. Pyrethrum formulation is based on a natural product as the active ingredient and is environmentally-friendly and, therefore, presents an opportunity for safe pest control.

Additional findings have also shown the possibility of colour of netting material being one of the significant physical attributes that can influence the knockdown effect of a treated net, although it did not significantly influence the kill effect on mosquitoes (Duchon *et al.*, 2006). The natural pyrethrum-formulation treated nets coloured blue and green achieved higher early knockdown than the pyrethrum-formulation-treated white nets. There is thus a possibility that coloured nets which undergo the dyeing process during manufacture become acidic during the clearing process and thus may blend more uniformly with the insecticides in the fiber, thus, enhancing contact toxicity resulting in early knockdown. Although needing further exploration, the findings seem to concur with previous observations (Duchon *et al.*, 2006), in which they observed that colour influenced the bio-efficacy of treated nets. The interaction between dose and netting colour in effecting knockdown, showed that bio-efficacy of a given dose impregnated on a net was influenced significantly by the net colour. These observations point out that the colour of a fabric may be important in deciding the impregnation target doses and eventual recommendation for effective ITNs that may be considered for large scale rollout.

In conclusion, the current study confirms the potential for use of the natural pyrethrum formulation in ITNs although treatment of nets should take into consideration the nature and colour of fabrics in use for the right doses to be determined and to maximize on the bio-efficacy potential of a net.

5.3 Distribution of *kdr* mutation in *An. gambiae* s.l. *and* susceptibility of the species with fixed *kdr* genes to the synergized pyrethrum formulation

Development of resistance by *Anopheles* mosquitoes to insecticides used in nets has the potential to seriously compromise the successful use of ITNs and malaria control in general (Chouaibou *et al.*, 2008; Yewhalaw *et al.*, 2011). The current study reveals that there are two key *Anopheles* sibling species i.e. *An. gambiae* s.s. and *An. arabiensis* with much higher dominance of the latter in the study areas. Results from the current study also showed that there were no recorded *kdr* in *An. arabiensis* but a fixed presence of *kdr* mutation genes in *An. gambiae* s.s. The occurrence of *kdr* L1014S to near fixation in *An. gambiae* s.s. observed in the current study is in agreement with previous observations conducted in different sites (Mathias *et al.*, 2011).

These observations are significant in planning malaria vector control initiatives since the two are part of the major Afro-tropical vectors that are efficient in transmission of *Plasmodium* parasites to human (Sachs and Malaney, 2002). The occurrence of *kdr* in *An. gambiae s.s* is of great concern to the use of ITN's because the species is known to exhibit anthropophilic and endophilic behaviour (Gillies and DeMeillon, 1968; Githeko *et al.*, 1994) and hence is most amenable to ITNs and indoor residual

spraying (IRS) which are the two key vector control technologies that have been shown to contribute significantly to malaria control efforts (Mnzava *et al.,* 1993; Donelly *et al.,* 1999).

Evidence of pyrethriod insecticides failing to control *An. gambiae* s.l. population with high levels of *kdr* resistance genes has been documented (N'Guessan *et al.,* 2007). The need therefore to develop new insecticide formulations targeting the emerging *kdr* resistance genes in *An. gambiae* is crucial. Given that the two species *An. gambiae* s.s. and *An. arabiensis* occur in sympatry (Magak, 2004), there is no ruling out the possibility that *An. arabiensis* may at one time also succumb to *kdr* mutation which might further complicate the whole vector control scenario. Indeed, high *kdr* frequency has been observed in populations of *An. arabiensis* in Ethiopia (Yewhalaw *et al.,* 2011) and Burkina Faso (Diabate *et al.,* 2004) resulting in negative effect in malaria vector control in those countries. Besides, among the *An. gambiae* complex, *An. arabiensis* is the most widely spread (Donelly *et al.,* 1999; Simard *et al.,* 1999). The lack of *kdr* resistance alleles observed in the species in the current study could be as a result of its exophilic behaviour that exposes it to less pyrethroids used indoors in ITN and IRS, the currently preferred vector control technologies in Kenya (Mnzava *et al.,* 1993; Donelly *et al.,* 1999). The significantly higher knockdown and mortality effect of natural pyrethrum-formulation on the mosquitoes with high levels of *kdr* genes suggest that the natural pyrethrum-formulation may have different resistance selection pattern to the pyrethroids. This is especially significant because the natural pyrethrum-formulation may then be used to "boost" the failing pyrethroid-based ITN's which have currently been rolled out to the masses since withdrawing them would be very costly and logistically untenable. This resistance scenario may be

anticipated especially deducing from the observed low knockdown and mortality of mosquitoes with *kdr* genes exposed to permethrin while the molecule has wide usage in ITNs and is an active ingredient in some of the most widely used brands of LLNs (www.olyset.com). Low knockdown effect with deltamethrin is equally of concern.

The mechanism of action of the natural pyrethrum formulation on *kdr* resistance genes may not at this point be explicit, but the fact that the natural pyrethrum-formulation had a significantly higher kill effect on mosquitoes than the unformulated natural pyrethrum may point to the role of the synergist and anti-oxidant that forms part of the formulation. Their addition may have other roles in influencing sensitivity of the site of action of the insecticides on the voltage gated sodium channel coupled with action on the activity of the mixed function oxidases that have previously been associated with pyrethroid resistance (Casida and Quistard, 1995; Hemingway *et al.*, 2004).

However, it is encouraging that *An. arabiensis*, still showed higher susceptibility to the natural pyrethrum formulation than permethrin implying that the natural pyrethrum formulation could still be applied in specific situations outdoors for its control using a variety of space-spraying techniques like fogging. The low knockdown levels exhibited by the pyrethroids on the *An. gambiae* s.l. may suggest emerging tolerance which may be enzyme-mediated for *An. arabiensis* and *kdr* for *An. gambiae* s.s. These observations are based on the complete absence of *kdr* genes in *An. arabiensis* and near fixed presence of *kdr* genes in *An. gambiae* s.s. observed in the current study.

The study has also shown the surging population of *An. arabiensis* in malaria prone areas which is a challenge to ITN and IRS currently being promoted given the exophilic behaviour of the vector. The success of ITNs is premised on the endophilic and anthropophilic behavior of the target vector (Githeko *et al.*, 1996b). The study has also shown the existence of *kdr* resistance genes in *An. gambiae* mosquitoes which are a key vector with preference to human biting and dwellings and this shows that *kdr* resistance pattern is complex and is undergoing through a dynamic process that varies with mosquito species and insecticides in use. There is thus need for deeper understanding of factors that govern population dynamics of the vectors, resistance, and vector susceptibility to various insecticides in relation to available options for control. The study has now provided an evidence-based demonstration of the ability of the natural pyrethrum formulation in controlling the *An. gambiae* s.l. species and standing out as a viable option for managing *kdr* resistance that is currently a threat to successful use of ITN to control the malaria menace. The product could thus be used to "boost" the treated nets that have been distributed in areas where pyrethroid resistance exist.

5.4 Behavioural effects of natural pyrethrum formulation treated on nets on wild vector mosquitoes

The use of matched experimental houses in terms of design and sizes, allowed us to assume same probability of blood-seeking mosquitoes that were available to enter the experimental houses thus allowing for comparisons. The predominant vector in the Ahero study area was undoubtedly *An. gambiae*.s.l. The observed population structure of the *An. gambiae* vectors in areas with higher numbers of *An. gambiae* s.l. relative to *An. funestus* is consistent with previous reports (Magak, 2004) who

recorded a 69.8% presence of *An. gambiae* s.l. against 30% for *An. funestus*. This stability may be related to adaptability of the species especially with reference to breeding habits and longevity of *An. gambiae* s.l. which survives for more than four gonotropic cycles as compared to *An. funestus* (Charlwood *et al.*, 2000.). This made the area suitable for assessment of entomological impact of the natural pyrethrum formulation treated nets under evaluation.

In their work, (Guillet *et al.*, 2001), asserted that the main alternative insecticides available for treatment of ITNs apart from the approved synthetic pyrethroids are carbamates and organophosphates. This observation however, has been challenged by the results of the current study which has adduced empirical evidence on the potential for usage of natural pyrethrum-formulation in ITNs. Besides, the use of the two compounds, given that they are cholinesterase inhibitors in their mode of action, may pose toxicological risk to humans (WHO, 1986). Due to rapid action of natural pyrethrins on mosquitoes and their known excito-repellent properties (Casida and Quistard, 1995), the houses fitted with bednets treated with natural pyrethrum formulation had highly reduced numbers of mosquitoes that rested indoors and reduced their feeding success thus impacting on their ability to transmit disease. It had previously been observed that excitation effects of the active ingredients in some insecticides used in bednets can result in movement away from treated surfaces (Taylor *et al.*, 1981). Mass-killing effect and reduced adult mosquito populations results in reduced vectorial capacity (Curtis and Mnzava, 2000). In a separate study, reduced indoor resting population of *An. gambiae s.l* and *An. funestus* was also observed in houses with permethrin-treated bednets (Mathenge *et al.*, 2001).

The observed high level of gravid females in the houses with untreated nets further point to the effect of reduced successful feeding and probably reduced longevity due to pyrethrins knockdown and eventual kill effect. In terms of proportions, there were more *An. funestus* collected in the houses than the *An. gambiae* s.l. probably as a consequence of the highly endophilic nature of the latter species as opposed to the *An. arabiensis* that has been observed to be dominant in the area (Magak, 2004). Emerging higher occurrence of *An. arabiensis* compared to its closely-related sibling species *An. gambiae* s.s. has also been observed in other studies (Bayoh *et al.*, 2010).

There seemed to be higher feeding inhibition by pyrethrins to *An. gambiae* than *An. funestus* and this may have been due to the behaviour of the latter, which is normally highly anthropophilic while the more zoophilic *An. gambiae s.l.* especially the *An. arabiensis* that was dominant in the study area may have been driven to feed on alternative hosts, especially domestic animals kept within the homesteads after sensing presence of insecticide-treated nets (Githeko *et al.*, 1994; Kaburi *et al.*, 2009). While the importance of insecticide-treated nets in vector control has been reported with various insecticide molecules (Lindsay *et al.*, 1989; Mbogo *et al.*, 1996; Gimnig *et al.*, 2003), the current study, for the first time, is confirming the potential for use of natural pyrethrum formulation in ITNs.

The superior ability of natural pyrethrum formulation-treated nets in reducing indoor resting population and fed mosquitoes than permethrin may provide a "*prima facie*" case for the ability of the natural pyrethrum formulation to have effect on resistant mosquitoes. Permethrin has been used for a longer time and cases of resistance to the molecule have been variously reported (Vulule *et al.*, 1999; Hargreaves *et al.*, 2000).

The challenge with the current use of natural pyrethrum formulation in ITNs lies with the usually noted non-compliance with re-treatment schedules (Maxwell *et al.*, 2003). These schedules are currently driving the need for re-invigorated formulations for use in long life treated nets (LLN), although re-treatment of nets after six months has been shown to have better impact on parasitaemia and anemia in pregnant women (Marchant *et al.*, 2001).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMEDATIONS

6.1 Conclusions

1. The dose of natural pyrethrum formulation impregnated on a net and duration after exposure of mosquitoes to the treated net are important in effecting KD and mortality. However, at periods between 15-60 minutes post application, no difference in KD results regardless of the dosage level.
2. The natural pyrethrum formulation has a good potential for use in ITNs, however, the treatment of nets should take into consideration the nature and colour of fabrics in use for the right doses to be determined and to maximize on the bio-efficacy potential of a net. In general the formulation is most suited for treatment of nylon and polyester nets but not cotton nets.
3. The natural pyrethrum formulation can sustain up to 3 conventional washings without compromising the bio-efficacy of the treated net and is recommended for impregnation of polyester nets at 500 mg/m^2 that gives a consistent acceptable level of bio-efficacy persistence of up to six months.
4. There were no mosquitoes with *kdr* mutation genes in Ahero and Kipsitet where only *An. arabiensis* was present, although in Kisian, the East African L1014S *kdr* mutation was present but only in the *An. gambiae* s.s. The natural pyrethrum formulation was, however, highly efficacious on both wild species from the different areas and the pink-eyed *An. gambiae* s.s. with *kdr* genes indicating the potential for use in managing *kdr* and other forms of resistance.
5. The higher reduction in the population of indoor resting and feeding in *An. gambiae* and *An. funestus* in houses fitted with bed nets treated with natural

pyrethrum formulation than in houses that had untreated nets shows the relative importance of treating nets with the formulation. The relatively higher level of gravid female mosquitoes in the houses with untreated nets than in houses fitted with nets treated natural pyrethrum-formulation, points further to the effect of increased successful feeding in houses with untreated nets than in houses with treated nets. Reduced feeding of mosquitoes in the holed nets treated with natural pyrethrum-formulation treated nets, compared to the untreated nets, shows the potential of natural pyrethrum-formulation treated nets to offer protection even in case of wear and tear of nets.

6.2 Recommendations

6.2.1 Recommendations for application of the current study

1. Bio-efficacy tests to assess knockdown (KD) effect on *Anopheles* mosquitoes could be standardized at 15 minutes post-exposure as opposed to 60 and 30 minutes currently recommended by the WHO and Pest Control Products Board (PCPB) of Kenya respectively in order to save on time and resources.
2. The use of bednets treated with natural pyrethrum formulation is recommended in nylon fabric at 320mg/m^2 and polyester nets at 500mg/m^2 in order to achieve maximum efficacy and exploit the safety and natural botanical profile of pyrethrum as a local solution to the malaria problem. It is however uneconomical to use the formulation in fabric nets cotton.
3. The fact that bednets treated with the natural pyrethrum formulation could sustain up to 3 washes without losing entomological competence, confirms its suitability for use in ITNs since under field use conditions, it has been established that, nets are not often washed more than two times within six

months. However because unwashed nets remained efficacious even at six months post installation, re-treatment of such nets should be considered only after the period. This would help in tackling the grave malaria problem with its devastating effects thus saving millions of vulnerable lives.

4. Even though there was lack of *kdr* mutation genes in *An. arabiensis*, in the study areas, for proper malaria vector control planning and management, there is still need for continuous monitoring of the spread of resistance conferring allele in this species, because among the *An. gambiae* complex, *An. arabiensis* is the most widely spread.

5. Susceptibility of *An. gambiae* with *kdr* genes to the natural pyrethrum formulation indicates that it's potential as a viable option to manage *kdr* resistance that is currently a threat to successful use of ITNs to control the malaria menace. In the event that the emerging resistance to pyrethroid insecticides in the LLN's becomes unmanageable, then a viable alternative would be the natural pyrethrum-formulation to be used for "boosting" the nets, since the nets are designed to last for many years and doing away with them would be costly.

6. High susceptibility of the *An. gambiae* s.l. to the natural pyrethrums formulation can increase the effectiveness of the ITNs especially when there is low net coverage of the population this is important because in most malaria control programmes, ITN coverage is often less than 50% so reduction of vector population remain an important strategy. The natural pyrethrum formulation has a "jamming" effect where mosquitoes exposed to it get confused and stop seeking blood meals and this jamming phenomenon opens up a new arena for natural pyrethrum formulation use in treated nets,

especially where the nets get torn or worn out. The superior ability of nets impregnated with natural pyrethrum formulation in reducing indoor resting population and fed mosquitoes than permethrin, may provide a "*prima facie*" case for the ability of the formulation to have effect on resistant mosquitoes.

6.2.2 Recommendations for future research

1. Research on stabilizing the natural pyrethrum formulation for use in LLN which is the current technology of choice to help consolidate the potential gains for using the new pyrethrum product in malaria control.
2. Undertaking basic molecular studies to understand the mechanism of action of the formulation on *kdr* resistance genes.
3. Further research to understand the reason for lack of *kdr* resistance genes in *An. arabiensis* as opposed to *An. gambiae* s.s. in same locality.
4. There is need for strong information, education and communication component in bednet usage because insecticidal effect is not usually appreciated as most users will prefer a clean net at the risk of losing entomological impact.
5. Further studies to understand the distribution, spread and contribution of *kdr* genes in ITN systems would advice further on the best control strategies.
6. Large scale longitudinal study on natural pyrethrum treated nets and the effect on malaria morbidity and mortality in human.

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