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Research Article

Dose-Effects of Bednets Impregnated with a Natural Pyrethrins Formulation: Persistence, Feeding Inhibition, and Wash Resistance against *Anopheles gambiae* s.s.

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Abstract *Background.* This study evaluated dose-response, persistence, irritancy and wash resistance of mosquito nets treated with a synergized natural pyrethrins-formulation against *Anopheles gambiae* s.s. *Methods.* Multi-filament polyester netting was impregnated to deposit 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. Dose-response, persistence, and wash resistance were determined by a 3-minute cone bioassay on three-day-old female *A. gambiae* s.s. and residual-insecticide persistence was assessed in modified WHO bioassay tunnels and HPLC, respectively. *Results.* Interaction between dose and time post-exposure to treated net on knockdown (KD) and mortality of mosquitoes was significant ($P = 0.0001$). Significant likelihood ratio ($\chi^2 = 33.23$, $P = 0.00266$) confirmed post-exposure time effect on KD. Unwashed pyrethrins-impregnated nets at 500 mg/m^2 had consistent KD and mortality of $> 90\%$ for six months. Nets washed 1–3 times achieved $> 85\%$ KD and mortality. A reduced efficacy was observed after wash 4 ($P = 0.0001$), with a 46% knockdown and 42% mortality. *Conclusion.* Bio-efficacy to assess KD on *Anopheles* could be standardized at 15 minutes post-exposure. Pyrethrins-formulation is suitable for nets impregnation due to its persistence (> 6 months) and allows for three washes without compromising bio-efficacy. Further work on improvement of the formulation to allow use of pyrethrins in long life nets is recommended.

Keywords dose effects, bednets, natural pyrethrins, *Anopheles gambiae* s.s.

1 Background

Malaria is still a leading cause of mortality especially in Africa where entomological inoculation rate (EIR), can exceed a thousand infectious bites per person per year [25]. The use of DDT and other synthetic insecticides in the 1960s for indoor residual spraying (IRS) to control adult mosquitoes was the first significant step in malaria eradication through vector control [14]. However, the escalating cost of IRS and logistical constraints have stimulated renewed interest in exploring other methods of vector control or selective spraying targeting areas of seasonal transmission [10,26]. It is estimated that the cost of full coverage in homes with children under the age 5 is $4.01 per person for two-treatment rounds with deltamethrin [33].

A cost-effective intervention with little vertical malaria control infrastructure required is the insecticide-treated nets (ITNs) [13,22]. The WHO pesticides evaluation scheme (WHOPES) recommends only insecticide formulations containing pyrethroid molecules, viz. alphacy-permethrin, cyfluthrin, deltamethrin, permethrin, and bifenthrin as the active ingredients in the insecticide formulations used in treatment of bednets [19]. Despite elaborate literature documenting the health impact of ITNS [1,8], there is not a corresponding effort in research into other alternative insecticide formulations suitable for ITNs. This scenario is worsened by the continued emerging resistance to pyrethroid molecules by *Anopheles* malaria vectors in many parts of Africa [20,24,27]. A potential molecule that can be exploited in the making of novel bednet formulation is pyrethrins derived from *Chrysanthemum cinerariaefolium*, a perennial temperate plant with small white daisy-like flowers. Currently, there is scanty documented evidence on the use of pyrethrins in ITNs despite its being a natural botanical insecticide that is environmentally friendly, with several advantages over chemically synthesized insecticides. Despite their photolability, when suitably formulated with a synergist and antioxidants, pyrethrins increase in their stability and residual efficacy [28].

Even though the current preferred technology is the usage of long life nets (LLNs), it is probable that, due to many inter-related factors, including resistance development, poor handling, wear and tear, the nets in usage may, at some point in time, require "boosting" to enhance their effectiveness and hence the re-treatment formulations that are being developed are key to tackling this imminent challenge. Besides, there are many nets still being manufactured and which will also need intermittent treatment making the current study critical in the evaluation of ITNs.

However, effective use of pyrethrins formulation in ITNs calls for understanding of the basic interrelations between the dose-response effects, feeding inhibition, residual persistence, wash resistance and the biological efficacy of the formulation against mosquitoes. Dose-response relationships fulfill an essential first step in formulating field treatment requirements and gives numerical measure of its effectiveness. This strategy can be used in structure-activity correlation, resistance measurements, and general decision-making, including optimizing the cost-benefit ratio of a product [18].

Successful feeding by mosquitoes is key to disease transmission since it is one of the possible outcomes of a host-female vector encounter. The irritant effect of certain insecticides can reduce mosquito-human biting habit in houses and divert mosquitoes to feed on domestic animals, sheltered near human dwellings [12]. Irritancy may further enhance the potency of a treated net in case of tear or wear under domestic usage. Thus, assessing the residual bio-efficacy of an insecticide on a treated net is crucial in determining the appropriate re-treatment period. To be fully effective, bednets need to be treated with insecticide once or twice a year [29]. Washing of a net directly impacts on the dosage of contactable insecticide remaining on it. In addition, it leads to an eventual reduction of the insecticide-dose to a level with minimal entomological impact [21]. Earlier studies have also shown that insecticide's active ingredients respond differently to washing. For example, it was observed that deltamethrin and permethrin were more wash-resistant than etofenprox and bifenthrin [22]. Therefore, this informs the need to evaluate response of specific active ingredients to washing. There is, however, scanty information on some performance parameters in relation to nets treated with pyrethrins formulation. As such, the aims of the current study were, to determine dose-response relationship, to assess the feeding inhibition, to determine the residual efficacy and to evaluate the effect of repeated washes on bio-efficacy of pyrethrins-treated nets against *Anopheles gambiae* s.s. mosquitoes to support appropriate use of pyrethrins-formulation in ITNs.

2 Methods

2.1 Formulation of pyrethrins-based emulsifiable concentrate (EC) for treatment of bednets

An emulsifiable concentrate (EC) formulation containing 5% pyrethrins, weight/volume (w/v), mixed with a synergist, an antioxidant and a non-ionic emulsifier, all put in a petroleum solvent base was made in the formulation laboratory at the Pyrethrum Board of Kenya (PBK) as per established procedures [2]. In addition, the active ingredient was obtained from 25% (w/v) pyrethrins pale extract batch-manufactured at the same processing factory. The synergist, an antioxidant and emulsifier were commercial grades obtained from the manufacturers, Endura Spa (Italy) and Bayer AG (Germany), respectively. After formulation, the active ingredient content was determined to be $5.0 \pm 0.5\%$ using a Varian Vista Series 5000® high performance liquid chromatography (HPLC). Briefly, 0.1 g of the sample was put in 100 mL volumetric flask and hexane added to the mark. The mixture was thoroughly shaken to achieve homogeneity, then 10 μL were injected into the HPLC system manually at the set flow rates of 8 mL/min, peak detector at 230 nm, column factory packed CN10, run time 10 min and chart speed at 0.25 cm/min. 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). Other physical parameters like flash point, specific gravity, water content and pH were also determined using standard procedures at the PBK Chemistry laboratory.

2.2 Impregnation procedure of the test mosquito net

A commonly used fabric in ITN, a multi-filament polyester netting of 100 deniers strength, with 20 holes/cm^2 (Siam Dutch mosquito netting company, Thailand), was purchased from local stores in Kisumu City and used in the current study. The nets were impregnated 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 [17]. 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. The amount of pyrethrins formulation in the stock solution was then determined and mixed with water in a non-absorbent plastic bucket. The nets were then carefully soaked in the mixed concentrate in a "dip-it-yourself" manner. 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. The quality of impregnation was assessed by cutting two pieces of the treated netting measuring 10 cm $\times$ 10 cm and put into a non-absorbent plastic bucket for instrumental chemical analysis at the PBK chemical laboratory using a

HPLC machine. Prior to analysis, the pieces of nets were weighed, soaked in 40 mL of hexane in a beaker. The extract was shaken for 10 min, then transferred into a volumetric flask connected to a condenser in which the sample was subjected to a hot extraction of pyrethrins for 8 hours. After extraction, 50 microlitres of the extract was injected into the HPLC machine for determination of the concentration of pyrethrins as described above. All netting pieces analyzed achieved a minimum of 95% of the targeted dose and, thus, ascertained the integrity of the impregnation process.

2.3 Test mosquitoes

Laboratory bioassay tests were carried out on susceptible three-day-old female *A. gambiae* s.s., Kisumu strain, reared at Kenya Medical Research Institute (KEMRI), Centre for Global Health Research (CGHR) laboratory. The test mosquitoes were bred as previously described [11].

2.4 Effect of pyrethrins-treated netting on knockdown and mortality of *An. gambiae* s.s.

Bioassays were conducted in accordance with the previously described WHO guidelines [30,31]. 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 a transparent plastic WHO-cones using clips. Twenty five (25) female *Anopheles* mosquitoes, aged 3 days, 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 after every 3, 5, 10, 15, 30, and 60 minutes. Thereafter, the mosquitoes were transferred into 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 treatment (75 mg/m^2, 100 mg/m^2, 250 mg/m^2, 500 mg/m^2, and 1000 mg/m^2) simultaneously under room temperature. Mosquitoes, exposed to untreated nets and bednets treated with permethrin at the recommended dose of 500 mg/m^2 were used as negative and positive controls, respectively.

2.5 Determination of dose-response relationship

The response of the mosquitoes in terms of knockdown and mortality in relation to the different doses of pyrethrins-treated nets was determined from the bioassays. A logit (i.e. log-logistic) regression analysis was used to study the insecticide dose-response relationship. The logit regression output was used to determine the various KD_{50} and KD_{95} doses while selectivity indices was used for comparison of the relative potency at LD_{50}.

2.6 Assessment of biological and chemical residual persistence of pyrethrins on impregnated nets

Residual persistence of pyrethrins on treated nets was monitored through bioassays performed on a monthly basis on the pyrethrins-treated nets at 250 and 500 mg/m^2 for 6 months as described above. Further evaluations were carried out monthly by chemical analysis, in which case, two pieces of treated nets, measuring 10 cm × 10 cm, were subjected to HPLC as described above.

2.7 Effect of washing on persistence of pyrethrins-treated nets

A multi-filament polyester netting fabric of 100 deniers strength was conventionally treated with pyrethrins at the recommended dose of 500 mg/m^2. The net was cut into several pieces measuring 22.5 cm × 22.5 cm before subjecting them to 1–5 washes using local bar soap (white star® bar soap) with soft water. In the washing process, 2.0 g of the soap was dissolved in 500 mL 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 10 times and rinsed twice with 500 mL 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 above, 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 Varian Vista Series 5000 HPLC as described above. The remaining 5 pieces (out of the 10) were used in bio-assays as per WHO procedures. The number of washes leading to over 80% mortality after 24 hours and 95% KD after 60 min post-exposure to the treated net was, then, determined.

2.8 Effect of pyrethrins-impregnated nets on feeding inhibition and irritancy of mosquitoes

Blood-feeding inhibition and irritancy of the mosquitoes by nets impregnated with pyrethrins at 250 and 500 mg/m^2 were studied in the laboratory based on a modified WHO tunnel test procedure [17,32]. In this test, non-blood-fed female mosquitoes, aged 3–5 days were released in a glass tunnel measuring 60 cm length, 25 cm high and 25 cm wide. The tunnel had three chambers, i.e. "release," "middle," and "baited." Each netting sample to be tested had 9 holes measuring 1 cm diameter each with one hole located at the center, while the other eight were equidistantly located at 5 cm from the border. A total of 400 cm^2 surface was available for possible contact with the mosquitoes. The sample to be tested was fixed on a cardboard frame and placed at the separation between the baited chamber (which held a rabbit in an immovable position) and the middle chamber. In the cage at the end of the longer section of the tunnel ("release chamber"), 50 female mosquitoes, in four replicates, were successively introduced and left to fly freely in an attempt to make contact with or reach the bait through the holes. The experiments were carried out at room

Table 1: Interaction between concentrations of pyrethrins impregnated on a net and time post-exposure on knockdown and mortality of *Anopheles gambiae* s.s.

Concentration (mg/m^2)	% KD 3 min ± SD	% KD 5 min ± SD	% KD 10 min ± SD	% KD 15 min ± SD	% KD 30 min ± SD	% KD 60 min ± SD	% mortality ±SD
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	10.07	9.17	7.89	5.25	6.47	6.76	8.12
P	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Data are ANOVA results showing the interaction effects of concentration of pyrethrins impregnated on nets (mg/m^2) and time post-exposure (% KD-3 min–% KD-60 min) on knockdown and % mortality of the test mosquitoes. Data are means unless otherwise stated. Concentration refers to the amount of pyrethrins-impregnated on nets expressed in mg/m^2 except for the standard permethrin at 500 mg/m^2 designated as 500 P. Data in columns show the proportion of mosquitoes that were knocked down after 3–60 minutes post-exposure. % mortality refers to the proportion of mosquitoes that were killed after 24 hours post-exposure. Means in the 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. SD = standard deviation.

temperature starting from 18.00 to 08.00 hours the following day. A total of 2 tunnels were used simultaneously, with one tunnel with nets impregnated with pyrethrins and the other tunnel with untreated netting (negative control). Counts were made on mosquitoes that accessed or were deterred from accessing the host. Blood-feeding inhibition was assessed using the following formula; (NC-NT)/NC × 100; where NC and NT refers to the number of blood-fed mosquitoes in untreated and treated tunnels, respectively.

2.9 Statistical analyses

Data on counts were expressed as percentages and where the coefficient of variation was more than 15%, the data were transformed into logarithms to stabilize the variance. Analysis of variance (ANOVA) of a randomized block design was carried out to partition variation into components for dose, time, and number of washes using SAS software (SAS Institute, Cary, USA). In cases where ANOVA table showed significant variations between treatments, least significant difference test (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 evaluate the insecticide dose-response relationship [6]. The logit regression output was used to determine the various KD_{50} and KD_{95} doses while selectivity indices was used for comparison of the relative potency at LD_{50}.

3 Results

3.1 Efficacy of pyrethrins-treated nets on knockdown and mortality of *An. gambiae* s.s.

Table 1 presents data on the efficacy of pyrethrins-treated nets on knockdown and mortality of *An. gambiae* s.s. Results reveal that, impregnation dose and time post-exposure were highly significant (df = 6, F-value = 1175, $P = 0.0001$; df = 5; F-value = 63.3; $P = 0.0001$, respectively) sources of variation in the levels of KD and mortality on the test mosquitoes (Table 1). The results further demonstrate that there was a general increase in KD and mortality with increase in dose. In addition, there was a highly significant (df = 30, F-value = 8.59, $P = 0.0001$) interaction between dose and time post-exposure. For example, at 3 min 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 (df = 6; F-value = 73.86; $P = 0.0001$). In terms of mortality, there were highly significant differences between the 75 mg/m^2 and 100 mg/m^2 doses (df = 6; F-value = 209.21; $P = 0.0001$). However, mortality was comparable between pyrethrins concentrations at 250 mg/m^2 and 500 mg/m^2, and between 500 mg/m^2 and 1000 mg/m^2 (Table 1).

3.2 Dose-response models and determination of lethal doses and effective/optimum dose on knockdown and mortality

Figure 1 presents the computed dose-response curves for each time along with the data points as derived from the logit regression output. The figure shows a general increase in rate of change in knockdown and mortality as post-exposure time increased, although this rate of decreased between 15–60 minutes resulting in a cluster. Taking LD_{50} as a base, there was no significant difference between KD-15 min and KD-30 ($P = 0.6312$), KD-15 min and KD-60 min ($P = 0.1590$), KD-30 min and KD-60 min ($P = 0.3400$). There was highly significant likelihood ratio ($\chi^2 = 33.23$, df = 14, $P = 0.00266$), implying that this

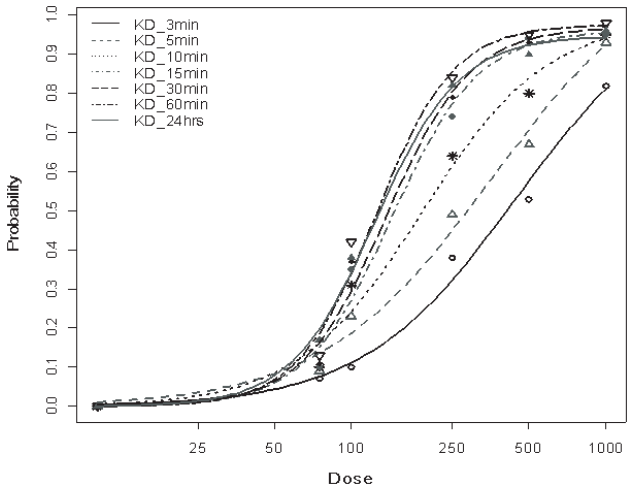

Figure 1: *Dose-probability response curves per time period and the respective data points.* Knockdown and mortality of *Anopheles gambiae* s.s. exposed to different doses of pyrethrins-treated nets shown as dose-probability models. Dose refers to the amount of pyrethrins impregnated on a net in mg/m^2. Probability values refer to % knockdown and % mortality of mosquitoes transformed into probability values. The legends KD-3 min, KD-5 min, KD-10 min, KD-15 min, KD-30 min, and KD-60 min refer to the period of observation of mosquito knockdown after exposure to various doses of pyrethrins-treated nets while KD-24 refers to mortality after 24 hours post-exposure.

model had a 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 353 mg/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 = 195.0–565.0), respectively.

3.3 Residual persistence of pyrethrins on impregnated nets

Results on bioefficacy of unwashed treated nets over a 6-month period are shown in Table 2. There was a significant difference (df = 6; F-value = 3.15; $P = 0.004$) between the treated and untreated nets in terms of KD at 60min post-exposure from 2 months after impregnation. However, significant difference (df = 6; F-value = 1.34; $P = 0.028$) in mortality was observed only at 6 months post-treatment. In general, both KD at 60 min and mortality after 24 hours remained stable at above 90% over the whole 6-month testing period. Chemical analysis of residual pyrethrins remaining on treated nets over a 6-month period is as shown in Table 3. It was observed that time (months) post-treatment significantly (df = 6; F-value = 24.97; $P = 0.0002$) affected the quantity of pyrethrins remaining on unwashed treated nets. A significant (df = 6; F-value = 24.97; $P = 0.0002$) reduction in quantity of pyrethrins was observed at 3 months post-treatment, with the lowest reduction recorded in the 6 months after impregnation. During this period, there was a 25% dose reduction from 5.09 mg/100 cm^2 to 3.81 mg/100 cm^2 (Table 2).

3.4 Effect of washing on persistence of pyrethrins-treated nets

Results on wash-resistance of pyrethrins-impregnated nets are shown in Table 3. It was observed that washing of pyrethrins-impregnated bednets significantly affected KD (df = 5; F-value = 156.32; $P = 0.001$) and mortality of mosquitoes. At the recommended treatment rate of 500 mg/m^2, the unwashed nets achieved a mosquito KD of 99% at 60 min post-exposure and mortality of 95% 24 hours post-exposure. This level of efficacy was comparable with that of the treated nets washed 1X (which gave 97% KD at 60 min and 96% mortality) and nets washed 2X (which achieved 96% KD and 93% mortality) 24 hours post-exposure (Table 3). However, a significant reduction in efficacy was observed after the nets were washed 3X (df = 5; F-value = 156.32; $P = 0.0001$), in which only 85% KD and 85% mortality were realized. Substantially reduced efficacy was observed when nets were washed 4X, in which case we only realized 46% KD and 42% mortality 60 min post-exposure. This was further reduced to 23% KD and 17% mortality after 5X washes.

Further chemical analysis revealed that there was a significant reduction in the amount of impregnated pyrethrins remaining on treated nets as the number of washes increased (Table 3). In addition, 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 3).

3.5 Effect of pyrethrins-impregnated nets on feeding inhibition and repellency of mosquitoes

The effect of treated nets on the mosquito feeding and repellency is shown in Table 4. Results reveal that dose of pyrethrins impregnated on a net significantly (df = 2; F-value = 7.82; $P = 0.013$) influenced the number of mosquitoes deterred from accessing the rabbit host through the simulated holes. The trend of deterrence of mosquitoes from accessing the host rabbit was as follows: 76% in the nets treated with pyrethrins at 500 mg/m^2, 68% in the nets treated with pyrethrins 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 (df = 2; F-value = 11.92; $P = 0.003$) affected the feeding success as follows: 11% feeding from the nets treated at 500 mg/m^2, 27% in the nets treated at 250 mg/m^2 and 86% in the untreated nets (Table 4). Of the mosquitoes that accessed the host through netting treated at 500 mg/m^2, only an average of 2 fed on the host blood as compared to

Table 2: Residual bioefficacy of nets treated with pyrethrins at 500 mg/m^2 on knockdown and mortality of *Anopheles gambiae* s.s. and chemical persistence over six months.

Month	% KD 3 min ± SD	% KD 15 min ± SD	% KD 30 min ± SD	% KD 60 min ± SD	% mortality ±SD	% pyrethrins on net ± SD	Quantity of pyrethrins (mg/100 cm^2) ±SD
0	$24.0^{cd} \pm 3.2$	$72.0^{a} \pm 2.4$	$90.0^{a} \pm 2.2$	$99.0^{a} \pm 0.9$	$98.0^{a} \pm 1.7$	1.18 ± 0.015^{a}	5.09 ± 0.066^{a}
1	$26.0^{bcd} \pm 4.6$	$61.0^{b} \pm 3.3$	$89.0^{a} \pm 1.7$	$92.0^{ab} \pm 1.7$	$60.0^{ab} \pm 1.4$	1.21 ± 0.022^{a}	5.15 ± 0.124^{a}
2	$21.0^{d} \pm 2.7$	$60.0^{bc} \pm 3.2$	$86.0^{a} \pm 2.2$	$92.0^{b} \pm 1.4$	$97.0^{a} \pm 0.9$	1.19 ± 0.03^{a}	5.14 ± 0.02^{a}
3	$37.0^{ab} \pm 4.1$	$55.0^{bc} \pm 5.0$	$87.0^{a} \pm 2.2$	$96.0^{ab} \pm 2.0$	$94.0^{ab} \pm 2.2$	1.06 ± 0.02^{b}	4.57 ± 0.104^{b}
4	$40.0^{ab} \pm 3.2$	$55.0^{bc} \pm 2.6$	$87.0^{a} \pm 3.0$	$95.0^{ab} \pm 1.7$	$94.0^{ab} \pm 1.7$	1.02 ± 0.02^{b}	4.33 ± 0.03^{bc}
5	$38.0^{ab} \pm 4.6$	$50.0^{c} \pm 2.2$	$86.0^{a} \pm 2.2$	$92.0^{b} \pm 1.4$	$94.0^{ab} \pm 2.2$	$0.96 \pm 0.03^{b,c}$	4.10 ± 0.104^{c}
6	$34.0^{abc} \pm 3.6$	$51.0^{bc} \pm 1.7$	$86.0^{a} \pm 2.2$	$92.0^{b} \pm 1.4$	$91.0^{b} \pm 1.7$	0.91 ± 0.025^{c}	3.81 ± 0.07^{d}
P	0.0230	0.0044	0.888	0.0040	0.0284	0.0001	0.0002
LSD	12.6	10.4	7.7	5.1	5.9	0.011	0.034

Data are means (± standard deviation) unless otherwise stated. Month refer to the period when bioassay tests were conducted on the pyrethrins-treated nets. Data in columns, for example % KD-3 min, % KD-5 min, % KD-10 min, % KD-15 min, % KD-30 min, and % KD-60 min show the proportion of mosquitoes that were knocked down after the respective minutes, following an initial exposure to pyrethrins-treated nets for 3 min. % mortality refers to the proportion of mosquitoes that died after 24 hours post-exposure. % 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/10 cm^2. Means in the same column with same superscript letter are not significantly different according to Least Significance Difference (LSD) test at probability level of 0.05. SD = standard deviation.

Table 3: Effect of washing netting fabrics treated with pyrethrins at 500 mg/m^2 on knockdown and mortality of *Anopheles gambiae* s.s. and residual persistence of pyrethrins.

No. of washes	% KD 3 min ± SD	% KD 15 min ± SD	% KD 30 min ± SD	% KD 60 min ± SD	% mortality ±SD	% pyrethrins on net ± SD	Quantity of pyrethrins (mg/100 cm^2) ±SD
0	$23.0^{a} \pm 1.9$ (4.9)	$64.0^{a} \pm 1.6$ (8.1)	$88.0^{a} \pm 3.7$ (9.4)	$99.0^{a} \pm 1.0$ (10.0)	$95.0^{ab} \pm 1.9$ (9.7)	1.18 ± 0.02^{a}	5.08 ± 0.07^{a}
1	$22.0^{a} \pm 2.6$ (4.8)	$57.0^{a} \pm 3.4$ (7.6)	$87.0^{a} \pm 1.9$ (9.4)	$97.0^{a} \pm 1.9$ (9.9)	$96^{a} \pm 2.8$ (9.8)	0.86 ± 0.03^{a}	3.61 ± 0.13^{a}
2	$25.0^{a} \pm 4.4$ (5.0)	$50.0^{a} \pm 4.2$ (7.1)	$81.0^{a} \pm 3.0$ (9.1)	$96.0^{a} \pm 1.6$ (9.8)	$93^{ab} \pm 3.0$ (9.7)	0.54 ± 0.01^{a}	2.19 ± 0.06^{a}
3	$6.0^{b} \pm 1.2$ (2.6)	$18.0^{b} \pm 4.2$ (7.1)	$35.0^{a} \pm 10.4$ (5.8)	$85^{b} \pm 1.9$ (9.3)	$85^{b} \pm 1.9$ (9.3)	0.38 ± 0.01^{b}	1.49 ± 0.05^{b}
4	$8.0^{b} \pm 1.6$ (2.9)	$10.0^{c} \pm 2.6$ (3.2)	$13.0^{c} \pm 1.6$ (3.6)	$46^{c} \pm 2.6$ (6.8)	$42^{c} \pm 3.8$ (6.6)	0.14 ± 0.01^{b}	0.54 ± 0.04^{bc}
5	$7.0^{b} \pm 2.5$ (2.7)	$8.0^{c} \pm 1.6$ (3.0)	$12.0^{c} \pm 1.9$ (3.7)	$23.0^{d} \pm 1.9$ (4.9)	$17.0^{d} \pm 1.9$ (4.2)	$0.01 \pm 0.0^{b,c}$	0.04 ± 0.0^{c}
P	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0002
LSD	7.7 (1.05)	9.3 (0.97)	14.4 (1.19)	5.6 (0.39)	7.9 (0.5)	0.011	0.034

Data are means (± standard deviation) unless otherwise stated. Netting color refers to the three polyester nets colored, white, Green, and blue that were impregnated with pyrethrins-emulsifiable concentrate. Data in columns represented by % KD-3 min, % KD-5 min, % KD-10 min, % KD-15 min, % KD-30 min, and % KD-60 min 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. Figures in brackets represent % values transformed into arcsine values. % 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/100 cm^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. SD = standard deviation.

a mean of 20 in the untreated nets, resulting in about 90% feeding inhibition. For the 250 mg/m^2 pyrethrins netting, there was an average of 4 mosquitoes that fed giving a feeding inhibition level of about 80%.

4 Discussion

The current study was designed with the main aim of determining dose-response relationship, assessment of the irritancy effect and the residual efficacy. In addition, we determined the effect of repeated washes on bio-efficacy of pyrethrins-treated nets against *A. gambiae* s.s. mosquitoes. Results presented in this study demonstrate that there was a significant interaction between dose and time post-exposure of mosquitoes to the treated net on knockdown and mortality ($P = 0.0001$). A significant likelihood ratio ($\chi^2 = 33.23$, df $= 14$, $P = 0.00266$) was observed pointing out that this model better describe the relationship between doses, time and effects. The slopes were not uniformly parallel indicating that the relative responses at different observation times were not similar. The reason for the observed response distribution may be due to inter-individual responses as a result of biological variability in the test mosquitoes. Lack of significant differences in LD_{50} as observed from the logit regression output demonstrates lack of additional effect on knockdown at 15, 30, and 60 minutes resulting in clustered trend lines. Consistent with the results in the ANOVA, this

Table 4: Effect of nets impregnated with pyrethrins on feeding behavior of *Anopheles gambiae* s.s.

Dose (mg/m^2)	% passage inhibition of mosquitoes ±SD	No. of mosquitoes fed	% mosquitoes fed ±SD	% 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-values	0.013	0.0001	0.003	
LSD (0.05)	14.22 (0.91)	4.05	23.6 (2.95)	

Data are means (± standard deviation) unless otherwise stated. Dose (mg/m^2) refers to the amount of pyrethrins 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 × 100; where NC and NT refers to the number of blood-fed mosquitoes in untreated and treated tunnels, respectively. 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 for percentages and absolute values for numbers. P-values represent the calculated statistic probability levels.

observation implies that there is no additional effect at durations longer than 15 minutes.

Although the significance of dose-response studies on insecticides has been observed in other studies [18, 29], to our knowledge, no studies to date have been carried out on pyrethrins treated nets. Currently, the WHO in their testing procedures on insecticide susceptibility bioassays recommends that a knockdown (KD) of 95% be achieved after 60 minutes post-exposure [32]. The current study does show that results obtained with KD at 15 minutes do not significantly differ with KD at 60 minutes, notwithstanding the dose. So effectively, the standards could be made to be KD_{95} at 15 minutes to save on time and resources.

In addition, unwashed nets impregnated with pyrethrins at the recommended rate of 500 mg/m^2 had a consistent KD and mortality of more than 90% mosquitoes for up to six months. There was a significant reduction in efficacy after wash 4, when 46% knockdown and 42% mortality were realized, while after 5 washes, there was only 23% knockdown and 17% mortality realized. A total of 76% of the test mosquitoes were deterred from accessing the host while out of those mosquitoes that accessed the host, only 11% fed on the host blood, demonstrating high irritancy effect of pyrethrins-treated nets on mosquitoes.

Formulation involves complex physical and chemical interactions that occur between various components. These synergistic interactions significantly influence the bio-availability, performance and stability of the active ingredient [7]. In the current work, the resultant pyrethrins-formulation demonstrated 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, however, the added emulsifiers usually have varying units of ethylene oxide to give pyrethrins a greater affinity for water [3].

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. Furthermore, the fact that the polymer chains in the polyester net have the same polarity as pyrethrins which are non-volatile and are high-boiling point esters [3], may enhance diffusion of pyrethrins into the net. Addition of the synergist in the formulation is of interest because its mode of action is thought to be through inhibition of mixed function oxidase and esterase enzymes in insects and, indeed, enzyme-mediated metabolic detoxification is known to play a role in pyrethroid resistance [23, 27]. This formulation could, therefore, have the potential to control resistant mosquito strains. Our laboratory is currently investigating the potential role of enzyme-mediated detoxification in the control of pyrethroid resistant mosquitoes.

The long persistence of the formulation on treated nets could be attributed to the action of both a synergist and an anti-oxidant. Some synergists have been shown to stabilize some insecticides in some instances, hence increasing the potential for bio-persistence of a formulation. Addition of an anti-oxidant enhances stability to the pyrethrum formulation since they act by binding the oxygen molecules and, therefore, making them unavailable for free radicals, in the process, reducing the oxidative process, the main degradation route in pyrethrins [3]. The anti-oxidant may also interfere with mixed-function oxidase enzymes produced by insects in response to an insecticide, thus increasing the potential 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 at least 6 months with an allowance of, at most, two washes before re-treatment [29]. Studies have also shown that nets re-treated in less than 6 months have better impact than those not re-treated [15]. In line with previous findings in which nets were generally washed 2 or 3 times per year [16], we have shown in the current study that the pyrethrins-treated nets could sustain up to three washes.

Even though the current preferred technology is the usage of long life insecticide treated nets (LLINs), it is

probable that, due to many inter-related factors, including resistance development, poor handling, wear and tear, the nets in usage may, at some point in time, require "boosting" to enhance their effectiveness.

In support of previous observations [21,22], we have also shown that washing significantly reduces efficacy of a treated net, although with differences in magnitudes depending on the insecticide used for treatment. Additional studies have also shown that insecticidal effect is not usually appreciated as most users will prefer a clean net at the risk of losing entomological impact [9]. Hence, there is an increasing need for strong information dissemination, education and communication component in bednet usage. In the current study, for example, it was observed that washing three times reduced the initial dosage of pyrethrins by 70.6% while in other studies, the same number of washings in traditional manner (using cow-fat soap), reduced initial dosages by about 85% for cypermethrin and 99.8% for pirimiphos methyl, with no detectable residues for deltamethrin [22]. The relatively low wash fastness of pyrethrins impregnated on nets may also be due to its low solubility in water and the polymer blend.

The observed dose-dependent, reduced feeding of mosquitoes in the holed-treated nets, compared to the untreated nets, does confirm the significance of pyrethrins treatment of nets. In the current study, we observed 86% feeding success in the untreated nets as compared to 28% and 11% for nets treated with pyrethrins at 250 mg/m^2 and 500 mg/m^2. In studies carried out elsewhere, 45–70% of the mosquitoes were able to feed on the host with untreated nets [4]. Differences observed in the current vs. previous studies may have been due to specific feeding and resting behavior 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 [3], coupled with volatile additives in the formulation. It is also thought that pyrethrins have a "jamming" effect whereby mosquitoes exposed to pyrethrins get confused and stop seeking blood meals [3]. This jamming phenomenon opens up a new arena for pyrethrins use in treated nets, since it has been observed that even if nets get torn or worn out through various handling and use situations, they are still capable of offering substantial protection to the user.

The study has also shown that washing significantly reduces efficacy of a treated net and this has been observed previously in other studies [17,22], although with differences in magnitudes depending on the treatment insecticide. Additional studies 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 [5], hence the need for strong information, education and communication component in bednet usage. In the current study, it was observed that washing three times reduced the initial dosage of pyrethrins by 70.6% while in other studies, the same number of washings in traditional manner, 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 [22]. The relatively low wash fastness of pyrethrins impregnated on nets may also be due to its low solubility in water besides the polymer blend. High mortality of more than 90% observed with the recommended dose of 500 mg/m^2 is crucial because in most malaria control programmes, ITN coverage is often less than 100% [16], so reduction of vector population remain an important strategy.

5 Conclusions

In this section, we demonstrate that the pyrethrins-formulation is suitable for impregnation of nets since it has good persistence of not less than six months on nets, and allows for a window-period of three washes without compromising bio-efficacy. In the event of wear or tear of nets due to varied domestic handling systems, the pyrethrins-treated nets, have good deterrence effect, while mosquitoes that access the host seem to loose the ability to feed, thus, minimizing their vector competence. Our findings further point out that bio-efficacy test to assess KD effect on mosquitoes should be standardized at 15 minutes post-exposure as opposed to the currently recommended 60 minutes in order to save on time and resources. These findings can be incorporated in real-life situations as an initial step to appropriate and effective usage of pyrethrins in ITNs, which is currently the pillar of malaria vector control. Indeed, successful implementation of ITN requires sound knowledge of susceptibility of the mosquito to the available insecticide compound under different conditions.

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