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Koech Peter Kiplang'at
Department of Biological Sciences, Chuka University, Kenya P.O. Box 109-60400 Chuka, Kenya
Email: kiplangatpk@yahoo.com
Tel: 0721609059

Richard W. Mwangi
School of Biological Sciences, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya
Email: richwam2002@yahoo.com
Tel: 0722791271

Correspondence:
Koech Peter Kiplang'at
Department of Biological Sciences, Chuka University, Kenya P.O. Box 109-60400 Chuka, Kenya
Email: kiplangatpk@yahoo.com
Tel: 0721609059

Repellent Activities of *Ocimum basilicum*, *Azadirachta indica* and *Eucalyptus citriodora* Extracts on Rabbit Skin against *Aedes aegypti*

Koech Peter Kiplang'at, Richard W. Mwangi

ABSTRACT

Vector borne diseases are the major public health problems in developing countries particularly in tropics. Essential oils from plants can provide the safe and biodegradable alternatives to synthetic repellents, but plant-based repellent formulations available in the market are not effective. The purpose of the study was to investigate mosquito repellent activities of Sweet Basil (*Ocimum basilicum*), Neem (*Azadirachta indica*) and Lemon Eucalyptus (*Eucalyptus citriodora)* extracts. Different concentrations of the extracts were tested for mosquito repellency on rabbit skin as the host of *Aedes aegypti*. Laboratory reared starved females were used for the tests and data collection was done by observational parameters based on frequency of mosquito landing and blood engorgement. Synergised Crude oleoresin extract of Pyrethrum and Ballet mosquito repellent® were included as positive test controls and Vaseline pure petroleum jelly® as a negative test control. The results showed that synergised Pyrethrum oleoresin showed complete protection at 0.1% as compared to Lemon Eucalyptus oil and Sweet Basil oil at 2% and 3% respectively($p<0.05$). Neem oil and Ballet did not provide complete protection. The mean percent repellency of 5% Neem oil was 84.21 and that of Ballet was 66.84 ($p<0.05$). Sweet basil and Lemon Eucalyptus oils can be alternative to Pyrethrum as natural mosquito repellents from plant origin.

Keywords: *Ocimum basilicum, Azadirachta indica, Eucalyptus citriodora,* Pyrethrum oleoresin, PBO, *Aedes aegypti,*

1. Introduction

Mosquito-borne diseases such as malaria, dengue hemorrhagic fever, encephalitis and filariasis are the major sources of illness and death worldwide, particularly in tropics [1, 2].

Malaria represents 9% of the total disease burden [1]. The major region affected by the disease is sub-Saharan Africa, where it causes at least 90% of the deaths reported [4]. Furthermore, an estimated 300-500 million new cases of infection are reported every year [1]. It also contributes to 25-40% of all out-patient visits and 20-50% of hospital admission and about 20% deaths of children under the age of 5 years [5] averaging at one child death in every 30 seconds [6]. The cost of its control is estimated at US$1.8 billion every year [3].

Dengue fever is endemic in more than 100 countries in Africa, the Americas and the Eastern Mediterranean and now it is a major health problem threatening an estimated 2.5 billion worldwide and 50-100 million new infections per year [7].

As a result of little success in the development of effective vaccines against these diseases coupled up with the problem of drug and insecticide resistance [7, 8] scientists have turned attention to mosquito control through exploitation of its behaviour [3]. Personal protection from mosquito bites has become a major approach to the control [9, 10, 11]. Application of repellents to the skin is a common personal protection practice [10] since it is a practical and an economical means of preventing transmission of these diseases to humans [8] besides providing protection against a wide range of vectors [12]. Repellents are substances that act locally or at a distance from the body, deterring an insect from flying to, landing on or biting human or animal skin [3]. It can also refer to molecules that may alter the functioning of sensory motor systems and or have neurotoxic effects [13].

They act either through air borne molecules as vapour of volatile oils or by non-volatile molecules with an antifeedant effect on the skin [3]. By their action, they minimize contact between man and insect vectors to hinder the transmission of diseases [14, 15].

DEET (N, N-diethyl-3-methylbenzamide), formally known as diethyl-m-toluamide, is currently the most effective synthetic repellent available in various commercial formulations such as solutions, gels, creams, aerosols, sticks and impregnated towellete [16]. However effective it is, toxic effects have been documented that include uncomfortable sticky skin, damage to plastics, synthetic clothes or rubber [17, 18, 19].

Moreover, continuous application can cause in folding of the skin epidermis with fewer hairs and thickened epidermis with more vascularity [20]. Another limitation is that it may be easily removed by perspiration and its efficacy decreases dramatically with rising out door temperature [6]. This has highlighted the search of alternatives from plant origin that contains safe and biodegradable chemicals [20, 21].

Plants that have been used as indigenous methods of insect control produces secondary plant compounds with insect feeding deterrent properties [22, 23]. The production of these compounds was due to co-evolution of the plants with insects [22]. Essential oils from plants commonly used as fragrances, food flavorings agents and beverages contain bioactive phytochemicals, which have potential for use in insect control [24]. Present in the essential oils, are monoterpenes, sesquiterpenes, diterpenes and triterpenes derivatives that possesses insect repellent activities [25, 26].

However, repellent effects of plant products are commonly lower in both efficacy and duration than that of synthetic repellents, primarily DEET. This calls for the search of new bioactive compounds. Of particular interest, is the search of essential oil-based compounds that are expected to supplement synthetic compounds [8, 16]

Sweet basil has been used traditionally as a food flavour, seasoning of food and as an insect repellent [27, 28]. Neem on the other hand has been used as an insect repellent and insecticide [29] has been reported as an insect antifeedant [30]. Eucalyptus has various ethno botanical uses including the used in cure of coughs, wounds and other skin ailment [31] and its crude extract has been reported to be a mosquito repellent [32].

Although the plants have been reported to have insect repellent properties, there is no substantive data to indicate their level of effectiveness and the concentration that offer complete protection from mosquito bites. The purpose of the study was to test and compare mosquito repellent activities of sweet basil (*Ocimum basilicum*), Neem (*Azadirachta indica*) and Lemon eucalyptus (*Eucalyptus citriodora)* extracts on rabbits.

2. Materials and Methods

This study was done using Insectaries and Laboratories of the School of Biological Sciences, University of Nairobi, Kenya.

2.1 Mosquito rearing

Aedes aegypti mosquitoes were reared in the Insectary at University of Nairobi located in the School of Biological Sciences. The colony has been bred continuously over the last twenty years without exposure to insecticides, repellents and pathogens.

The eggs stored on filter papers were obtained from a desiccator. These were used to develop a temporary colony for the study. The eggs were then floated in rearing trays, half filled with tap water. At larval stage, they were fed daily with 100 mg of active dry yeast that was sprinkled on the water surface. Water in rearing trays was refreshed every two days in order to avoid scum formation that might kill the larvae. Trays were washed in clean tap water and the larvae sieved out of the trays and cleaned thoroughly with tap water before being returned to the fresh water in rearing trays. On pupation, the pupae were collected using a Pasteur pipette and transferred in a container, three-quarters full of water that was then inserted in a wooden cage.

The emerged adults were continuously provided with 10% sugar solution as an energy source. The prepared solution was placed in a feeding tube, Whatman No. 1 filter paper inserted and placed in the cage. The solution was changed every three days to avoid fermentation and growth of moulds.

The female adults used for the egg production were given access to blood meal from the blood vessels of the rabbit ears *ad libitum*. A live rabbit was restrained in a wooden box; head inserted in a mosquito cage and mosquitoes were allowed to feed for one hour. Whatman No 1 filter paper rolled in a cone-shape was inserted in a plastic container, three-quarter full of tap water and provided to the engorged females. Every three days, the eggs were collected and dried on a soft cotton wool before being stored in the desiccator until when required.

The colony was maintained at 25±2 ºC, 80±10% relative humidity and 12h: 12h (light: dark) photoperiod.

2.2. Test mosquitoes

The mosquitoes used for the laboratory repellent bioassay were 3-7 day old, laboratory-bred and starved adult females of *Aedes aegypti*. Prior to the time of tests, they were starved by being provided with only water for 18-24 hours.

2.3. Test rabbits

Experimental rabbits 2 males and 2 females were kept at room temperature and light: dark regime of (12L: 12D) in cages in the animal house located at the School of Biological Sciences in the University of Nairobi. They were fed daily on Rabbit pellets, Wheat bran, vegetables and water provided *ad libitum*. Beddings that consisted of wood shavings and grass was periodically changed.

2.4 Preparation of the test materials

2.4.1 *Ocimum basilicum*

Ocimum basilicum was collected from Athi River in Machakos district. The plant was uprooted and transported in polythene bags. The voucher specimen was deposited in the herbarium of the Department of Botany, University of Nairobi.

Fresh leaves were picked from the plant, washed with clean tap water before being dried under the shade to a constant weight. The drying was done slowly to avoid the loss in biological activity.

Fully dried materials were pulverized into fine powder by use of a hammer mill until the powder passed through 1mm mesh sieve. The fine powdered material was stored at room temperature before extraction. 1 kg of the plant material was then weighed and soaked in hexane for four days in a plastic bucket.

The mixture was constantly stirred with a wooden rod over that period. It was then decanted leaving soluble hexane fraction. The fraction was evaporated in a Rotary Evaporator ® at 60 ºC leaving behind *Ocimum* essential oil.

The crude extract obtained was dried, precipitated and crystals of camphor were removed. The resultant oil extract was collected,

placed in a white-coloured plastic container before being stored in a cupboard at room temperature until investigated for its repellent activity.

2.4.2 *Azadirachta indica*
Ripe fruits of *Azadirachta indica* were obtained from Rea Vipingo farm, Kilifi, North Coast. They were picked from the plant and placed in polythene bags. The voucher specimen of the plant was deposited in the herbarium of the Department of Botany, University of Nairobi.
The seeds whose fruit pulp had been removed were washed in clean tap water, shade-dried to a constant weight, and then stored at room temperature until the time of extraction.
Fully dried seeds were dehusked and the neem oil was pressed from seed kernels using an oil expeller. The extract obtained was sieved and filtered in order to remove solid particles. Neem oil extract was then kept in a white-coloured plastic bottle and stored at room temperature until the time of the test.

2.4.3 *Eucalyptus citriodora*
Eucalyptus oil was kindly provided by BIOP Company Limited ready for formulation. A sample containing the test extract was kept in a light green glass bottle at room temperature until evaluated for repellent activity.

2.4.4 *Chrysanthemum cinerariefolium*
Crude oleoresin extract consisting of 25% w/w of pyrethrins and Piperonyl butoxide (PBO), was kindly donated by Pyrethrum Board of Kenya at Nakuru ready for formulation. The test samples were tightly sealed in a transparent glass bottles and were kept at room temperature away from sunlight until required for repellent investigation.

2.5 Formulation of the test extracts
The test extracts were formulated using Vaseline Pure Petroleum Jelly® of Uniliver Kenya Limited. In preparing each test concentrations, plant extracts were volumentarily diluted in melted jelly to an appropriate concentration.
Different concentrations of the oil were prepared by diluting the test material in melted jelly using well-labelled hypodermic syringes. The jelly was scooped using a spatula and transferred into a clean 100 ml beaker. The beaker was then partially immersed in a water bath at 80 ºC then stirred continuously using a stirring rod until it was fully melted.
Using an appropriate syringe, a given volume of the test extract was transferred into test tubes in a rack partially immersed in the water bath, arranged and labelled according to prepared concentration. Fully melted jelly was then sucked using a different syringe and diluted with the extract to an appropriate concentration. The test tubes containing the test material was shaken and poured into respective labelled containers. They were then left to stand until they solidify and stored at room temperature until tested for repellent activity.

2.6 Laboratory evaluation of the test substances for mosquito repellency on rabbit skin
Fifty female mosquitoes were aspirated randomly using a mouth aspirator and the males were left to starve to death. The females were counted by observation through a transparent glass that makes the tip of the pipette before being transferred into mosquito cups.
Mosquito cups that were used were made from transparent plastic containers, which were obtained from the market. The top of a bottle-top was cut off and closed with a net. However, the bottle-top could still be opened or closed. A circular hole was created at the centre of the cup and a tube-like net attached to it that could be tied or untied. Mosquitoes were introduced to the cup through the centre-hole and at the end of the experiment removed by opening the bottle-top besides it acting as a feeding area.
After the introduction of mosquitoes to the cups, they were then left to acclimatize for one hour before the commencement of the experiments. Tests for mosquito readiness to feed were done by holding a mosquito cup that contained the female mosquitoes on rabbit ears. As soon as mosquitoes were observed to land and attempt to feed, the cup was withdrawn before imbibing. Mosquitoes was then allowed to feed on the untreated skin and the sample that showed high percentage feeding were used for the tests (Figure 3 and Table 1).
Since *Aedes aegypti* feeds during the day, all tests were conducted between 09:00 h to 16:00 h. The test rabbit was restrained in a wooden box and test areas were shaven using a shaving machine. Different concentrations of test extracts were applied on the shaven area and rabbit ears. The cup containing the test mosquitoes were labelled according to the test substance and held on the test area for one hour. After each test, the test areas were cleaned with unscented soap. All the experiments were done in the experimental room in light and at room temperature. Observations was done through the plastic container was done and the effects of the test substances on the mosquitoes during the experiments was recorded.
At the end of the experimental period, mosquitoes were anaesthetized by placing cotton wool dipped in ether on top of experimental cups. The bottle-tops were then opened and mosquitoes were poured on the filter paper. The numbers that fed were observed sorted out and confirmed by pressing against a filter paper to produce a blood smear. Those that had a blood were considered to have taken a rabbit blood meal.
They were then counted and used in determining percentage repellence that was then calculated as follows [33].

$$\mathbf{C\text{-}T/C \times 100}$$

Where C is the number of mosquitoes that fed on the untreated skin and T is the number of mosquitoes that fed on the treated skin.

2.7 Petroleum jelly
A placebo that consisted of Vaseline Pure Petroleum Jelly ® of Uniliver Kenya Limited was also included as negative control. The petroleum jelly was purchased in the market and stored at room temperature until the time of the experiments.

2.8 Ballet
Ballet ® mosquito repellent of BIOP Company Kenya Limited contains olibanum oil, eucalyptus oil, geranium oil and citronella oil and it is currently sold in Kenya in most retail outlets was also included as positive test control.

2.9 Data analysis
Data analysis was done using Ms Excel and Graph pad prism 4 for windows. Two-way ANOVA followed by Bonferroni post-test was performed in order to compare repellent activities of different concentrations of each of the test extracts.
One-way ANOVA followed by Tukey test was performed in order to determine whether there were differences in mosquito repellent activities of each of the test extracts and their controls.

In order to compare the different formulations and their controls, one way ANOVA was performed followed by Tukey test. A *p* value of less than 0.05 was considered to indicate statistical significance.

3. Results

Effects of the test extracts on mosquito behaviour

Ocimum basilicum oil and *Eucalyptus citriodora* oil were observed to have high mosquito repellent activity with the emergence of mosquito paralysis at concentrations of 3% and 1% respectively giving 100% and 99.34%, respectively.

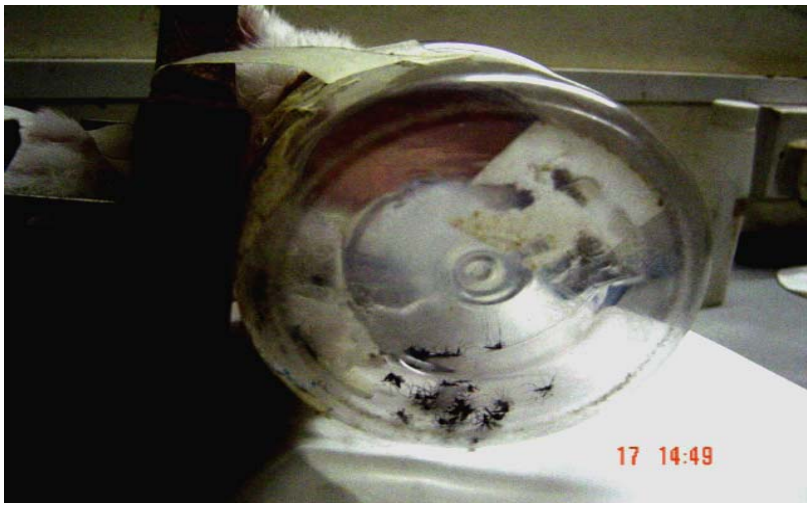

Fig 1: A photograph showing *Aedes aegypti* female mosquitoes paralyzed after application of Sweet basil oil on the rabbit ear (side view)

Fig 2: A photograph showing *Aedes aegypti* female mosquitoes paralyzed after application of Sweet basil oil on the rabbit ear (Front view)

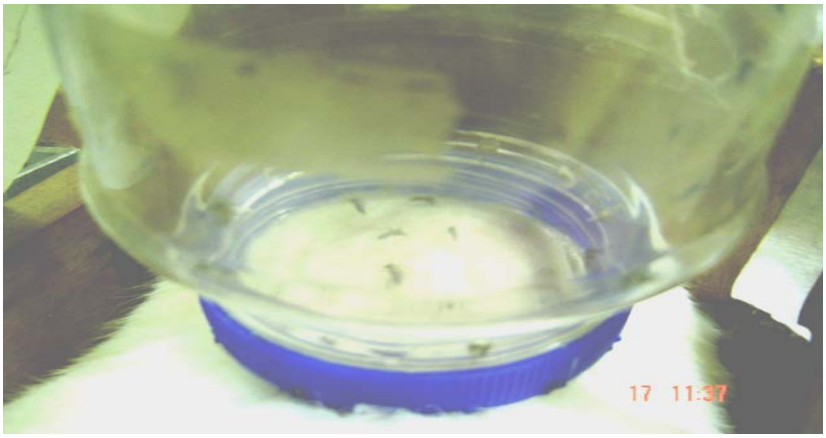

Fig 3: A Photograph showing *Aedes aegypti* female mosquitoes feeding on untreated rabbit skin

Synergised pyrethrum (*Chrysanthemum cinerariefolium*) which is known for that action in insects displayed the effect at 0.1%. However, at concentrations less than these no such activity was observed. Nevertheless, no such activity on the skin treated with *Azadirachta indica* oil was noted at all the tested concentrations.

However, the results obtained on 30 random samples of mosquitoes tested on untreated skin indicate high feeding activity with a mean feeding of 76% (Table 1) and were observed not to display any change in their normal behaviour (Figure 3). Similarly, no such activity was observed with Vaseline® Pure Petroleum Jelly and Ballet.

Table 1. Feeding activity of *Aedes aegypti* females on untreated rabbit skin over a period of 30 days

Tests (days)	Fed	Unfed	Total	Percentage fed
1	27	13	50	54
2	29	11	50	58
3	35	15	50	70
4	40	10	50	80
5	41	9	50	82
6	45	5	50	90
7	46	4	50	92
8	47	3	50	94
9	48	2	50	96
10	48	2	50	96
11	49	1	50	98
12	30	20	50	60
13	31	19	50	62
14	33	17	50	66
1 5	35	15	50	70
16	37	13	50	74
17	42	18	50	84
18	42	18	50	84
19	44	16	50	88
20	47	13	50	94
21	29	21	50	58
22	30	20	50	60
23	30	20	50	60
24	35	15	50	70
25	37	13	50	74
26	37	13	50	74
27	38	12	50	76
28	46	14	50	92
29	30	20	50	60
30	34	16	50	68
Mean ±SE	38.07±1.268	12.93±1.101	50±0.0	76.13±2.537

Table 2: Percent repellent activity of Neem oil, Sweet basil oil, Eucalyptus oil and crude oleoresin extract of pyrethrum against starved *Aedes aegypti* females

	Neem oil		Sweet basil oil		Eucalyptus oil	
Conc. (%)	**Mean±SE**	**95% CI (L,U)**	**Mean±SE**	**95% CI (L,U)**	**Mean±SE**	**95% CI (L,U)**
1.0	53.29 ± 14.79	6.24, 100.30	74.35 ± 7.92	49.13, 99.56	99.34 ± 0.66	97.25, 101.40
2.0	51.97 ± 13.60	8.68, 95.27	91.45 ± 5.19	74.92, 108.00	100.00 ± 0.00	100.00, 100.00
3.0	53.29 ± 8.35	26.72, 79.86	100.00 ± 0.00	100.00, 100.00	100.00 ± 0.00	100.00, 100.00
4.0	65.79 ± 11.11	30.42, 101.20	100.00 ± 0.00	100.00, 100.00	100.00 ± 0.00	100.00, 100.00
5.0	84.21 ± 4.81	68.92, 99.50	100.00 ± 0.00	100.00, 100.00	100.00 ± 0.00	100.00, 100.00

Pyrethrum oleoresin (*Chrysanthemum cinerariefolium* extract) + PBO		Vaseline Petroleum jelly		Ballet mosquito repellent		
Mean± SE	**95% CI (L,U)**	**Mean± SE**	**95% CI (L,U)**	**Mean± SE**	**95% CI (L,U)**	**P value**
100.00 ± 0.00	100.00, 100.00	13.68 ± 1.75	8.84, 18.53	66.84± 10.77	36.93, 96.75	0.05
100.00 ± 0.00	100.00, 100.00	13.68 ± 1.75	8.84, 18.53	66.84± 10.77	36.93, 96.75	0.05
100.00 ± 0.00	100.00, 100.00	13.68 ± 1.75	8.84, 18.53	66.84± 10.77	36.93, 96.75	0.05
100.00 ± 0.00	100.00, 100.00	13.68 ± 1.75	8.84, 18.53	66.84± 10.77	36.93, 96.75	0.05
100.00 ± 0.00	100.00, 100.00	13.68 ± 1.75	8.84, 18.53	66.84± 10.77	36.93, 96.75	0.05

Table 3. Percent repellent activity of *Ocimum basilicum* oil, *Eucalyptus citriodora* oil, *Azadirachta indica* oil and crude oleoresin (*Chrysanthemum cinerariefolium* extracts) against starved *Aedes aegypti* female

Test extract	Concentration				
	0.0001%	**0.001%**	**0.01%**	**0.1%**	**1%**
Neem oil *(Azadirachta indica*	13.16	18.42	15.79	15.79	55.26
Sweet basil oil (*Ocimum basilicum*)	23.68	26.32	55.26	71.05	78.95
Lemon eucalyptus oil *(Eucalyptus citriodora)*	31.58	55.26	78.95	89.47	97.37
Pyrethrum oleoresin *(Chrysanthemum cinerariefolium* extract*)* + PBO	75.44	85.09	93.86	100	100
Ballet Mosquito Repellent®	89.47	63.16	60.53	31.58	89.47
Vaseline Pure Petroleum Jelly®	13.16	15.79	18.42	13.16	7.89

4. Discussion

The plant extracts were tested to possess mosquito repellent activities and the effects were dose-dependent ($p<0.05$). Synergised *Chrysanthemum cinerariefolium* extract has a high repellent activity followed by *Eucalyptus citriodora oil*, *Ocimum basilicum* oil and *Azadirachta indica* respectively (Table 2 and Table 3). However, there was no significant difference between pyrethrum extract in comparison to *Eucalyptus citriodora oil* and *Ocimum basilicum* oil ($p>0.05$). *Eucalyptus citriodora oil* and *Ocimum basilicum* oil was more effective than Ballet $p<0.05$). Neem was less effective as compared to pyrethrum extract ($p<0.05$) but as effective as Ballet ($p>0.05$).

Neem oil was not effective at concentrations of 0.0001%, 0.001%, 0.01% and 0.01% ($p>0.05$) (Table 3). At this point, an increase in concentration resulted in no significant change in repellent activity. However, it is effective at a concentration of 1% and above. An increase in concentration to 4% led to a remarkable change in repellent activity. However, it was not as significant as compared to 1%, 2% and 3% concentrations ($p>0.05$). At a concentration of 5%, there was a marked improvement in the repellent activity and the activity was significant when compared to concentrations of 1%, 2% and 3% but it was not as significant as compared to the concentration of 4 % (Table 2). Furthermore, the results obtained at the concentration of 5%, (Table 2) agree with the of 5% neem oil in cream formulation as a topical application on humans [34]. It is also noted that percentage repellency of the concentration was higher as compared to that of Ballet ®. Moreover, no concentration tested of these extract provided a complete protection (Table 2).

Results obtained from the extract from *Ocimum basilicum* indicated a high repellent activity. A complete protection offered in mice as earlier reported [35] was consistent with the present results. However, the oil was not effective at 0.0001% and 0.001% but effective at 0.01% and above (Table 2 and Table 3). At a concentration of 2%, percentage repellency was observed to be 91.45, which is higher than that of Ballet®. Complete protection was obtained at 3% of the oil, but beyond this concentration, there was no significant change in the activity (Table 2). Furthermore, mosquito paralysis occurred at this concentration. This activity is similar to the reported effect of DEET [36, 13]. This shows that the extract is most likely to be having neurotoxic effects on insects and it fits the description of a repellent by [13]. Though effective, the limitation of this extract is the strong smell which can be improved when used in synergy with other extracts. The insecticidal activity of the extract cannot be ignored.

Lemon eucalyptus oil was not effective at the concentration of 0.0001% and 0.001% but was effective at a concentration of 0.01% and above (Table 2 and Table 3). There was an increase in activity with an increase in concentration of the extract. However, at 1% and above, an increase in concentration did not lead to a significant change in repellent activity. It was however noted that there was no change in activity beyond 2% (Table 2). A complete protection was found to be 2%. Furthermore, it was found that its percentage repellency was higher than that of Ballet at a concentration of 0.01% that was 78.95 (Table 2).

Synergised crude oleoresin extract of pyrethrum was tested to be effective at 0.0001% (1ppm) and complete protection was obtained at 0.1% (Table 3).In this concentration, there was no difference with 2% and 3% *Eucalyptus citriodora* oil and *Ocimum basilicum* oil respectively (Table 2 and Figure 3). It is however noted that at a concentration of 0.0001%, percentage repellency was obtained to be 75.44 and this was higher than that of Ballet (Table 2 and Figure 3). However, the results did not agree with the field studies which show percentage repellency as 96 of 50% pyrethrum oleoresin extract in coconut oil [37]. The synergy with PBO is most likely to have improved its activity.

Ballet® does not provide complete protection with the mean repellency of 66.84 (Table 2). Based on these results, the claim that Ballet® protects up to 8 hours is false. Vaseline Pure Petroleum Jelly also showed some activity since it was observed to be at 13.68 (Table 2). The activity of Vaseline is most likely due to the odour of the Jelly. The low activity of Ballet might be due to the low concentration of *Eucalyptus* used. This also shows that synergy does not exist between the plants extracts used in the formulation.

5. Conclusions

Ocimum basilicum and *Eucalyptus citriodora* can be an alternative to *Chrysanthemum cinerariefolium* extract. Increasing the concentration of Eucalyptus oil to 2% in Ballet and including 3%

Ocimum basilicum oil is most likely to improve its efficacy. Further research should be carried out to determine the bioactive compounds present in *Ocimum basilicum* responsible for the repellency. Field activity and test on primates is recommended. The duration of time taken by the test extracts should also be investigated. The need to conserve *Ocimum basilicum* is therefore advised.

6. Acknowledgements

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