International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2016; 3(4): 06-10

Received: 02-05-2016
Accepted: 03-06-2016

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Repellent and feeding deterrent activity of a natural formulation from plant extracts on rabbit and human skin against *Aedes aegypti*

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Abstract
Aedes aegypti mosquito females transmit diseases such as Dengue, Yellow fever, Zika fever and Chikungunya associated with mortality in many developing countries. Zika fever has neither treatment nor vaccine. Personal protection by application of the skin repellents is the major control approach. Though synthetic skin repellents are effective, they are toxic however the natural mosquito repellents such as Ballet® are not effective. The purpose of the study was to develop a natural repellent formulation that can provide 100% repellency. A formulation was developed from a combination of *Azadirachta indica, Eucalyptus citriodora*, *Ocimum basilicum* and *Chrysanthemum cinerariaefolium* extracts. They were applied thinly on rabbit ears and tested with starved *Aedes aegypti* females. Observations were made on the effect of the test combination, frequency of mosquito landing and blood engorgement. The combination that worked on rabbits was then tested on human skin of volunteers. The formulation provided complete protection, had mosquito paralytic effect and had higher repellent activity than Ballet® ($p<0.001$). The final formulation that may scale up and applied for human use is a combination of *Azadirachta indica, Eucalyptus citriodora, Ocimum basilicum* and *Chrysanthemum cinerariaefolium* extracts.

Keywords: Mosquito-borne diseases, Natural repellent formulation, *Aedes aegypti,* Ballet®

1. Introduction
Aedes aegypti female mosquitoes transmit viral diseases such as dengue hemorrhagic fever [1], yellow fever [1]Zika fever [2] and chikungunya fever [3]while seeking for human blood meal [4]. There is evidence that Zika fever is associated with intrauterine growth restriction including abnormal brain development in their fetuses, miscarriage [5] or microcephaly [6, 7]. It also is a link to neurologic conditions in infected adults [3].Recently, there is a reported outbreak of chikungunya fever in some parts of Kenya had resulted in deaths and hospitalization [8]. Globally, Yellow fever causes an estimated 200,000 new infections and 30,000 deaths every year [9] of which nearly 90% of the cases occurring in Africa [10]. Since 1950s, Zika fever has been known to occur within a narrow equatorial belt from Africa to Asia but recently it has spread to Central America, the Caribbean and South America where it has reach pandemic levels [11]
There is no vaccine or drugs against Zika fever [12] and no cure against yellow fever [13]. Thus major approach of mosquito control is through personal protection such as sleeping insecticide-treated bed nets [14] and application of insect repellents [15]. Repellents can significantly lessen the chance of contracting vector-borne diseases [16] and are particularly useful when used where human activity coincides with the diurnal activity patterns of mosquitoes such as outdoor activities that take place at dusk and dawn [17]. More over, *Aedes aegypti* bites during day time [18] thus application of repellents to the skin is the most practical, economical, convenient and effective way of protection from mosquito bites [19, 20].
Synthetic repellents such as N, N-diethyl-3-methylbenzamide, (DEET) are the most effective but allergic and toxic effects have been documented [21, 22]. Moreover, it may be easily removed by perspiration and its efficacy decreases dramatically with rising out door temperature [20]besides damaging plastics, synthetic clothes or rubber [23].This has calls for the need for the search of natural repellents from plants that are equally effective and contains safe and biodegradable chemicals [24] such as essential-oil based compounds [25].
Azadirachta indica extracts have been tested to have various effects on insects such as an insecticide, growth regulator and antifeedant [26] and repellent [27].

On *Aedes aegypti* mosquitoes, when tested on exposed body parts of human volunteers, 5-40% of neem oil in coconut oil offered 85% protection [28] and neem cream containing 5% neem oil offered 84% protection [29]. *Ocimum basilicum* essential oil provides protection from bites from *Aedes aegypti* for 75 minutes [30] and protects up to 30 min by on the human-bait technique using the standard WHO method [31]. *Eucalyptus citriodora* protects for 1 hour against on *Aedes aegypti* mosquitoes [32]. It was found that a lemon-eucalyptus oil containing p-menthane-3, 8-diol has similar efficacy compared with DEET repellents [33]. Oleoresin of pyrethrum (*Chrysanthemum cinerariaefolium*) when mosquito population that consisted mainly of *Mansonia* in a field study and found that it offered 96% protection at 50% concentration in coconut oil [33].

The purpose of the study was to develop a natural repellent formulation from *Azadirachta indica, Eucalyptus citriodora, Ocimum basilicum and Chrysanthemum cinerariaefolium* and test it on rabbit and human skin.

2. Materials and Methods

2.1. Study area

The study was done in the insectaries and laboratories located in the school of biological sciences, University of Nairobi.

2.2 Test mosquitoes

Mosquitoes used for the study were nulliparous, 5-7 day old female *Aedes aegypti* mosquitoes. The colony has been bred continuously for over twenty years without exposure to insecticides, repellents and pathogens. They was maintained at 25±2 ºC, 80±10% relative humidity and 12h: 12h (light: dark) photoperiod.

The eggs stored on filter papers were floated in plastic rearing trays, half filled with tap water. The emerged larvae were fed daily with 100mg of active dry yeast. Every 24 hours, water was refreshed. The pupae were picked using a Pasteur pipette and transferred in a container, three-quarters full of water that was then inserted in a wooden mosquito cage measuring 30 by 30 by 30 cm.

The emerged adults were continuously provided with 10% sucrose solution in Whatman No. 1 filter paper and that was changed every three days. 24 hours after emergence the adult females used for developing a temporary colony were fed with blood from the vessels of the rabbit ears *ad libitum*. Experimental female mosquitoes were not allowed to take a blood meal and were given only water 24 hours prior the experiment.

2.3 Test animals

Five New-Zealand white rabbits of either sex were used for the tests. They were kept in cages in the animal house at room temperature and light: dark regime of (12L: 12D). 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.

Five fourth year students of either sex drawn from Physiology class of the school of Biological sciences, University of Nairobi volunteered in the study. The Informed consent was sought and ethical approval was granted by University of Nairobi animal use and ethics committee.

2.4 Preparation of test formulation

Ocimum basilicum whole plants were collected and transported in polythene bags. The leaves were removed, washed with clean running tap water and shade-dried to a constant weight.

They were then pulverized into fine powder by use of a hammer mill until the powder passed through 1mm mesh sieve. 1 kg of the powdered material was soaked in hexane for four days, constantly stirred over that period and then decanted leaving soluble hexane fraction. The solvent was then evaporated in a Rotary Evaporator ® at 60 ºC leaving behind the essential oil which was then dried, precipitated and crystals of camphor removed.

Fresh ripe fruits of *Azadirachta indica* were collected and transported in polythene bags. They were washed in clean tap water and dried under the shed to a constant weight. The oil was pressed from de-husked seed kernels using an oil expeller, sieved and filtered to remove solid particles. *Eucalyptus citriodora* oil was kindly provided by BIOP Company Limited ready for formulation. Crude oleoresin extract of *Chrysanthemum cinerariaefolium* was donated by Pyrethrum Board of Kenya ready for formulation.

The test formulation was developed by mixing different amounts of essential oils extracts in melted Vaseline Pure Petroleum Jelly® of Uniliver Kenya Limited based on the results of previous experiments [34]. Different combinations was first tried that consisted of 5% *Azadirachta indica* and 2% *Ocimum basilicum, 5% Azadirachta indica* and 2% *Eucalyptus citriodora, 2% Eucalyptus citriodora* and 2% *Ocimum basilicum.* Ultimately, a combination consisting of 10% *Ocimum basilicum,* 10% *Eucalyptus citriodora* and 10% *Azadirachta indica* was tested. The latter combination was then reinforced with crude oleoresin extract of *Chrysanthemum cinerariaefolium* at 1mg/ml.

The test extracts were sucked using different syringes into respective test tubes and mixed thoroughly before they volumentarily diluted with melted pure petroleum jelly to the desired concentrations. The prepared formulations were then poured into respective labelled containers, left to stand to solidify and stored at room temperature until repellent investigations.

2.5 Test of developed formulation on rabbit skin

Ten females were aspirated, introduced into mosquito cups and left to acclimatize for one hour, prior to commencement of the experiment. Tests for readiness to feed were confirmed by holding the mosquito cup on the untreated rabbit ear and as soon as mosquitoes landed and attempted to feed the cup were withdrawn.

The test formulations were then smeared thinly rabbit ears. The right ear was for treatments and the left ear was used testing the controls. After each test, the test areas were cleaned with unscented soap and left to dry. The cups were labelled according to test formulation and then held on treated areas for 15 minutes. Observations were taken the number of mosquito landing and the effects of test substances on mosquito behaviour. At the end of the experiment, mosquitoes were anesthetized by placing a cotton wool soaked in Ether on the top of the cup. They were then poured and those fed were observed and confirmed by pressing against the filter paper.

2.6 Test of developed formulation on Human skin of volunteers

Formulation that worked on rabbits was tested on human skin of volunteers. 10 females [20] were aspirated randomly from the cage and introduced into the mosquito cups. Before the commencement of the tests, they were left to acclimatize in the

cup for one hour. Test for mosquitoes' readiness to feed was confirmed by inverting the cup containing the mosquitoes on untreated skin.

A test repellent sample and controls were then smeared thinly on a small area of volunteer's forearm. The right arm was used for the treatment and the left for controls. Volunteers were instructed not to touch, rub or wet the treated area. The cup labelled according to the test formulation was then inverted over the treated area for fifteen minutes. Observations on the mosquito landing and behaviour were taken during the experiment. After the experiment, test mosquitoes were anaesthetized in cotton wool dipped in ether that was placed on the top of the cup. Mosquitoes were then poured and pressed against the filter paper.

An ABO blood group test kit was used in order to determine the blood meal source and mosquito biting potential. Blood-fed mosquitoes were then pressed on three spots on the slide. Drops of human anti A and anti B were placed on the respective blood spots. They were then mixed with the help of a wooden applicator. The slide was then rocked back and fourth and observation on the on whether there is presence or absence of agglutination was then noted.

2.7 Statistical Analysis

The results were expressed as mean ± SEM .Statistical analysis were done by using one-way ANOVA followed by Tukey's multiple comparison test using Graph pad prism and Ms Excel for windows. A *p* value of less than 0.05 was considered to indicate statistical significance.

3. Results and Discussion

3.1 Effects of the formulations on mosquito behaviour

The new formulation showed a high activity with emergence of mosquito paralysis. However, no such effect was observed in untreated skin, Ballet® Mosquito Repellent and Vaseline® Pure Petroleum Jelly.

3.2 Tables and figures

Fig. 1: Test for mosquito readiness to feed on the rabbit ear

Fig 2: Mosquito cup containing *Aedes aegypti* female mosquitoes

Table 1: Percent feeding and landing of *Aedes aegypti* on a natural formulation-treated human skin under laboratory conditions

	Landing	Feeding	Repellency	*P* Value
	0.00	0.00	100.00	<0.05
	0.00	0.00	100.00	<0.05
	0.00	0.00	100.00	<0.05
	0.00	0.00	100.00	<0.05
	0.00	0.00	100.00	
Mean± SE	0.00±0.00	0.00±0.00	100.00 ± 0.00	<0.05
95% CI of the mean (L,U)]	0.00 ±0.00	0.00 ±0.00	100.00, 100.00	<0.05

Table 2: Percent feeding and landing of *Aedes aegypti* on a natural formulation-treated Rabbit skin under laboratory conditions

	Developed formulation			Ballet Mosquito repellent		Petroleum jelly		
	Landing	Feeding	Repellency	Landing	Feeding	Landing	Feeding	*P* Value
	0.00	0.00	100.00	30.00	30.00	80.00	80.00	<0.05
	0.00	0.00	100.00	40.00	40.00	70.00	70.00	<0.05
	0.00	0.00	100.00	50.00	50.00	90.00	90.00	<0.05
	0.00	0.00	100.00	30.00	30.00	80.00	80.00	<0.05
	0.00	0.00	100.00	20.00	20.00	80.00	80.00	<0.05
Mean± SE	0.00±0.00	0.00±0.00	100.00 ± 0.00	34.00±5.10	34.00±5.10	80.00±3.16	80.00±3.16	<0.05
95% CI of the mean (L,U)]	0.00	0.00	100.00, 100.00	19.85, 48.16	19.85, 48.16	71.22, 8.78	71.22, 8.78	<0.05

Table 2: Percent feeding and landing of *Aedes aegypti* on a natural formulation-treated Rabbit skin under laboratory conditions

	Developed formulation			Ballet Mosquito repellent		Petroleum jelly		
	Landing	**Feeding**	**Repellency**	**Landing**	**Feeding**	**Landing**	**Feeding**	***P* Value**
	0.00	0.00	100.00	30.00	30.00	80.00	80.00	<0.05
	0.00	0.00	100.00	40.00	40.00	70.00	70.00	<0.05
	0.00	0.00	100.00	50.00	50.00	90.00	90.00	<0.05
	0.00	0.00	100.00	30.00	30.00	80.00	80.00	<0.05
	0.00	0.00	100.00	20.00	20.00	80.00	80.00	<0.05
Mean± SE	0.00±0.00	0.00±0.00	100.00 ± 0.00	34.00±5.10	34.00±5.10	80.00±3.16	80.00±3.16	<0.05
95% CI of the mean (L,U)]	0.00	0.00	100.00, 100.00	19.85, 48.16	19.85, 48.16	71.22, 8.78	71.22, 8.78	<0.05

3.3 Discussion

The developed formulation displayed antifeedant and antilanding effect with no variation in both on rabbit and human skin ($p>0.05$). The activity of the formulation was higher than that of Ballet® ($p<0.001$). This is as a result in the difference in the composition and concentration of each formulation and the amount of synergy exists among its components. This formulation consisted of *Ocimum basilicum* oil, *Eucalyptus citriodora* oil, *Azadirachta indica* oil (1:1:1; each 10%) and 1mg/ml crude oleoresin extract of *Chrysanthemum cinerariaefolium* while Ballet consisted of Ballet contains olibanum, eucalyptus, geranium and citronella oils. The compounds present in each of the extract responsible for the activity include p-methane-3, 8-diol (PMD), azadirachtin and pyrethrins in *Eucalyptus citriodora, Azadirachta indica* and *Chrysanthemum cinerariaefolium* respectively. However, bioactive compounds present in *Ocimum basilicum* are not known.

The purpose of combining the extracts was because each had a role in the overall efficacy of the formulation. *Eucalyptus citriodora* plays a crucial role by providing an air barrier due to high volatility, *Ocimum basilicum* enhances paralytic activity, azadirachtin in neem oil deters a mosquito from feeding should it land on the skin and pyrethrins disrupts the coordination [35] and also effective at low concentration [34] thus reducing the amount of each extract making the formulation economically viable.

The volunteers did not complain of local skin reaction such as rash, swelling, irritation, or other allergic responses however there was complains of smell of the test formulation which is attributed to *Ocimum basilicum* in the formulation[36] . Thus, formulation can be improved by addition of citronella oil which is in perfumery and an effective repellent against *Aedes aegypti* [37].

Though this test formulation had a higher activity than Ballet® repellent, it is not known how effective as compared to DEET. The formulation may not provide complete protection for 8 hours after personal application due to reported high volatility of the plant essential oils [38] and effective for a relatively short period [39]. However it may be improved by addition of base or fixative materials, such as vanillin that has been tested to improved longevity of mosquito repellents a period of 6 to 8 h depending on the mosquito species [40].

The test extracts formulation can be used in mass production of mosquito repellent lotions, jellies and creams that can be incorporated to integrated vector control programmes. It also has an advantage of being formulated into sprays, vaporizers, sticks, candles and electrically heated dispensers that can be used in household protection from mosquito bites and other biting arthropods of medical importance

4. Conclusion

The claim that Ballet repellent protects up to 8 hours is false. The formulation can replace ballet however further investigations are needed to test its efficacy under laboratory conditions using arm in cage method and in the field for its commercial application.

Acknowledgements

I wish to acknowledge Pyrethrum Board of Kenya and BIOP Company limited for provision of the test materials.

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