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Synergistic repellent activity of plant essential oils against *Aedes aegypti* on rabbit Skin

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Abstract
Mosquito-borne diseases are the major causes of mortality particularly in tropics. Due to drug and insecticide resistance, personal protection by use of skin repellents has become a common approach of control. The purpose of the study is to determine if synergy exists between *Ocimum basilicum*, *Azadirachta indica* and *Eucalyptus citriodora* oils. Kinga Mosquito repelling Wax® and Vaseline Pure Petroleum Jelly® were included as positive and negative test control respectively. The results showed that *Chrysanthemum cinerariefolium* extract had no paralytic effect at 0.002% and 0.005% with mean repellency of 81.58 and 85.94 respectively. Similar observation was shown by 10% *Azadirachta indica* oil and Kinga with a mean repellency 85.79 and 80.53 respectively. *Azadirachta indica* oil was then reinforced by addition of Sweet basil oil and Lemon eucalyptus oil. A combination that provides complete protection and displayed mosquito paralysis was obtained. The developed formulation can replace Kinga® and alternative to *Chrysanthemum cinerariefolium* extract.

Keywords: Plant essential oils, Synergy, PBO, Percent repellent activity, *Aedes aegypti*.

1. Introduction
Mosquito females are nuisance blood sucking insects that transmits diseases such as Yellow fever, Dengue fever, Malaria, Japanese encephalitis and Filariasis while seeking for a human blood meal [1]. They transmits to more than 700 million people each year and remain the major source of illness and death world-wide [2] contributing to poverty and mortality thus affecting socio-economic development in tropical and subtropical countries [3]. Dengue fever is the most common life threatening viral infection with no proper vaccine or treatment [4]. It is transmitted by bite of infected *Aedes aegypti* [4] and affects 50 million people a year and 2.5 billion at risk [5].

Aedes aegypti bites during the day-time, indoors or in sheltered areas near houses [6] and the major target of control is by targeting the nervous system through behaviour modification [5] of which, personal protection from mosquito bites by application repellents to the skin is the common approach [7].

N, N-diethyl-m-tolnamide (DEET) is a wide-spectrum and most effective synthetic repellent available in the market [8]. However, allergic and toxic reactions after application have been documented [9] as well as demage to synthetic clothes, plastics and rubber [10]. Moreover, its continuous application can cause infolding and thickening of skin epidermis with fewer hairs [11].

Plant essential oils are potential natural repellents that are expected to replace synthetic compounds [12]. It contains monoterpenes such as a-pinene, cineole, eugenol, limonene, terpinolene, citronellol, citronellal, camphor and thymol that have mosquito repellent activity [13]. They are obtained from non woody parts of the plant particularly leaves and their individual compounds due to natural synergism that discourages development of resistance [14]. They are commonly used as fragrances, food flavours, confectionary, beverages, pharmaceuticals [15] and are considered non-toxic to humans [16].

Neem (*Azadirachta indica*) oil is non-toxic, non-irritating to the skin [17], considered safe for human health and environment [18]. The oil is derived from seeds and consists of triterpene azadirachtin [19]. It is non-volatile [20] with antifeedant [21] and repellent [17] effects against mosquitoes.

Sweet basil (*Ocimum basilicum)* oil is extracted from the leaves and flowers in superficial glands and trichomes [22] and consist mainly camphor and limonene [48]. It has been tested as

repellent againt *Aedes aegypti* [24, 25]. Though it offers complete protection and displayed paralytic effect [24] but, certain characteristics such as volatility [26] and strong odour [24] may limit its effectiveness.

Lemon eucalyptus (*Eucalyptus citriodora)* oil consist of citronellal, citronellol and PMD (p-methane 3, 8 diol) [27]. It has been tested as a repellent against *Aedes aegypti* [24, 28] however it is effective for a short duration of time [29] since it acts on mosquitoes in vapour- phase [30].

Chrysanthemum cinerariaefolium, a mixture of pyrethrin esters (Pyrethrin I and II, jasmolin I and II, and cinerin I and II) is known to have a knock-down, repellent and paralytic effects [31] in insects, but have a short residual activity [32]. Piperonyl butoxide (PBO) is used as its synergist but toxic effects have been reported [33].

If a volatile compound is combined with a non-volatile substance, it is possible to block insect attack both on the air and the skin surface [34]. Improved repellent activity and economic viability of neem oil could be improved by combining with Sweet basil and Lemon eucalyptus oils which are effective at low concentration [24].

Many researchers have also reported improved repellency effective over several hours with addition of a base or fixative materials, such as liquid paraffin [35].

The purpose of the study is to determine if synergy exists between Neem oil, Lemon eucalyptus oil and Sweet basil oil and compare the developed formulation with Pyrethrum extract synergised with PBO and the essential oil-based natural mosquito repellent formulation available in the market in Kenya.

2. Materials and Methods

2.1 Test mosquitoes

Aedes aegypti were reared in the Insectary of University of Nairobi, Kenya. The eggs in filter papers were placed plastic rearing trays, half filled with tap water.

The hatched larvae were fed daily with active dry yeast. Newly emerged pupae in a container, three-quarters full of water, was transferred in a screen cage.

The emerged adults were continuously fed with 10% sucrose solution and given access to blood meal from the blood vessels of the rabbit ears *ad libitum*. Prior tests, 3-7 day old females were aspirated from the cage, placed in mosquito cups and starved by giving only water for 18-24 hours.

The colony was maintained at 25±2 ºC, 80±10% relative humidity.

2.2 Test rabbits

New Zealand white rabbits 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.

2.3 Preparation of plant extracts

Healthy leaves of Sweet basil (*Ocimum basilicum)* were collected from Athi River, Machakos district. They were washed with clean running tap water and shade-dried to a constant weight.

Fully dried materials were 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. The crude extract obtained was dried, precipitated and crystals of camphor were removed.

Fresh ripe healthy fruits of neem (*Azadirachta indica*) were obtained from Rea Vipingo farm, Kilifi, North Coast. The fruit pulp were removed and washed in clean tap water, shade-dried to a constant weight. The oil was pressed from de-husked seed kernels using an oil expeller, sieved and filtered to remove solid particles.

Lemon eucalyptus (*Eucalyptus citriodora*) oil was kindly provided by BIOP Company Limited ready for formulation.

Crude oleoresin extract of Pyrethrum (*Chrysanthemum cinerariefolium*) and Piperonyl butoxide (PBO) was kindly donated by Pyrethrum Board of Kenya at Nakuru ready for formulation.

2.4 Formulation of the test extracts

The test extracts were formulated using melted Vaseline Pure Petroleum Jelly® by volumentarily diluting with melted jelly in water bath at 80 °C to the desired concentrations.

Different combinations that consisted of Neem oil and Sweet basil oil, Lemon Eucalyptus oil and Neem oil and Lemon Eucalyptus oil, Neem oil and Sweet basil oil in the ratio 1:1:1 were then prepared.

Crude oleoresin extract of pyrethrum was synergised with PBO in a ratio of PBO to pyrethrum as 4:1 (v:v) and the resultant stock solution was serially diluted to the desired concentration.

2.5 Test for repellent activity

Fifty females were randomly aspirated before being transferred into transparent plastic mosquito cups and then left to acclimatize for one hour before tests. Tests for mosquito readiness to feed were done by holding a mosquito cup that contained the female mosquitoes on the rabbit ear. As soon as mosquitoes were observed to land and attempt to feed, the cup was withdrawn before imbibing.

All tests were conducted between 09 00 h to 16 00h in light and at room temperature. The test rabbit was restrained in a wooden box and the ears and two shaven parts of the skin served as test areas. Different concentrations of test extracts and their combinations were applied on the test areas and the mosquito cups with the test mosquitoes held on the test area for one hour

Mosquitoes were then anaesthetized in ether and engorged mosquitoes were sorted, counted and confirmed by pressing against a filter paper.

A constant number of mosquitoes feeding on untreated skin were obtained by conducting tests over a period of 30 days using different batches.

The percentage of repellency was calculated by the formula.

Percentage repellency = **[(Ta - Tb)/Ta] x100** [36].

Where;

Ta is the mean number of mosquitoes on the untreated skin

Tb is the number of mosquitoes that fed on the treated skin.

2.6 Statistical analysis

Analysis was done using Ms Excel and Graph pad prism 4 for windows. One way ANOVA followed by Tukey test were used to compare the formulations and their controls. A *p* value of less than 0.05 was used to indicate statistical significance.

3. Results and Discussion

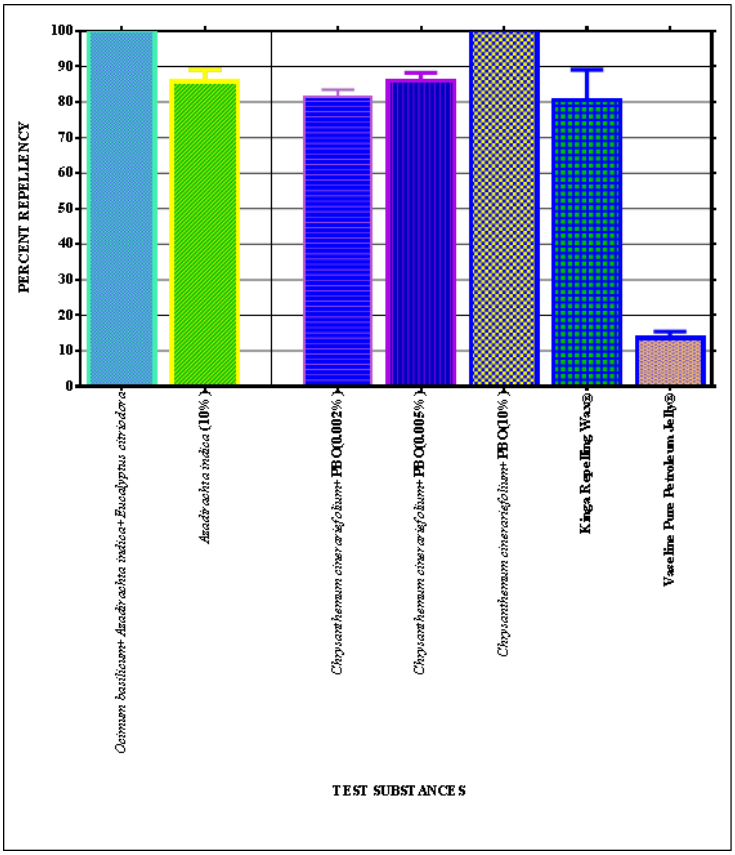

Fig 1: The repellent activity of the combination of the plant oils, *Azadirachta indica oil*, *Chrysanthemum cinerariefolium* extract and Kinga Mosquito Repelling Wax® against starved *Aedes aegypti* under laboratory conditions.

Increasing the concentration of neem oil from 5% to10% did not lead to any significant change in repellent activity with a mean repellency of 85.79 (Fig 1) as compared to 84.21 at 5% in the previous test[24]. This activity was higher than that Kinga and 0.002% Phyrethrum extract having a mean repellency of 80.53 and 81.58 respectively (p>0.05) (Fig 1).

The activity of Pyrethrum extract was dose-dependent. Increasing its concentration from 0.002% to 0.005% resulted in a slight increased repellency (p>0.05). This activity was higher than that of Kinga (p>0.05) (Fig 1). At a concentration of 10%, there was significant change in activity and mosquito paralysis was observed. The paralytic activity is due to the action of pyrethrins on central and peripheral nervous system resulting in disruptions of signal transmission along the nerve axon [31]. However, at low concentration below 0.01%, it led to mosquito avoidance reaction due to loss of coordination [31]. PBO acts as synergist by inhibiting the Mixed Function Oxidase system also known as the cytochrome P-450 system, a primary route of detoxification in insects that causes the oxidative breakdown of pyrethrins thus making it effective with less active ingredient [33]. Inspite of this, the reported toxic effects associated to PBO [33] and photosensitivity of phyrethrins [37] makes the formulation less effective as a skin repellent.

An ideal repellent as that which can provide initial mosquito anti-landing effect by providing an air barrier of volatile molecules and a secondary skin surface barrier [34]. Thus, mosquitoes that penetrate the air barrier of volatile molecules are further deterred if they meet a compound that is relatively non-volatile but possesses antifeedant effect on the skin [34].

Neem oil is antifeedant [21] and non-volatile [20] and its reinforcement with sweet basil oil and Lemon eucalyptus oil resulted in a superior product due to synergy of the extracts because of different components present in each which have different mode of action. The combination may also reduce the amount of each extract that could have been used to achieve the desired result thus making it economically viable. The formulation had higher percent repellency as compared to 10% neem oil alone and Kinga (p<0.05) (Fig 1) besides resulting in mosquito paralysis. The activity was also higher than that of 0.002% and 0.005% Pyrethrum extract.

The activity of neem oil is due to azadirachtin that acts by stimulating specific deterent cells in mosquito chemoreceptors and blocks the firing of sugar receptor cells which normally stimulate feeding [36]. Thus, it plays a very important role from deterring mosquito from feeding if it land on the skin. Sweet basil oil had similar paralytic effect as pyrethrum extract although it is effective at higher concentration than pyrethrum [24]. The exact mode of action of sweet basil oil is still

unknown and its activity is most likely due to camphor and limonene known for repellent activity. No such paralytic effect was observed in the skin in Vaseline, neem, and Kinga treated skin. Thus sweet basil oil serves to paralyze mosquitoes and block them on the skin surface since it acts in a vapour-phase. Eucalyptus oil had similar effect as sweet basil whose activity is most likely due to citronellal, citronellol and PMD (p-methane 3, 8 diol). It plays a role in potentiating its activity, neutralizes the reported strong smell of sweet basil oil [24] and may prolong protection time as previously reported [38].

Formulation in form of a jelly might have helped in improving its efficacy due to the activity of Vaseline Pure Petroleum Jelly. This could be due to the presence of liquid paraffin and other components present in the jelly (Fig 1).

4. Conclusion

Kinga Mosquito Repelling Wax® cannot reliably protect humans. The developed formulation can be an alternative but duration of protection and test on human skin of volunteers is necessary for its commercial use. Addition of vanillin is most likely to further improve its efficacy.

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