IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

Effectiveness of a plant-based mosquito repellent formulation

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Abstract Diseases transmitted by mosquitoes cause human morbidity and mortality and also lead to economic loss and social disruption in the poorest countries of the world. Repellents reduce mosquito host interaction, bites and associated distress. Many repellents in use today are expensive, of synthetic origin, have been associated with adverse effects and toxicities. Extensive use of chemicals has resulted in environmental toxicities, non-acceptance by users and resistance by the insects. Plants and plant products have repellent activities, do not persist in the environment and do not have major effects on non-target organisms and plants. They can substitute synthetic insecticides or complement the use of the same. The aim of this study was to develop a plant-based mosquito repellent formulation that is safe and efficacious to use. The plants from Msambweni subcounty, Kwale county, Kenya were selected for the study on the basis of ethnomedicinal and literature review of plants of on their use against mosquitoes. The plants' extracts were made into two formulations of 10% (formulation A) and 20% (formulation B). Acute dermal irritation and corrosion was assessed using New Zealand white rabbits according to OECD test guideline number 404. Repellency tests were performed according to the protocol of world health organization (WHO) using *Aedes aegypti* mosquitoes on human volunteers. The WHO bioassay method was used to determine knockdown effects of the formulations against mosquitoes. The two formulated products did not exhibit dermal irritation/corrosion on the rabbit or human skin. They exhibited a repellence effect greater than or similar to DEET. They can offer suitable substitutes to most synthetic repellents.
Key words: acute dermal irritation, repellent, knockdown effect, formulation, mosquito borne diseases

INTRODUCTION

Diseases transmitted by mosquitoes cause morbidity and mortality in humans. Mosquitoes are vectors of many diseases such as malaria, chikungunya, Zika virus and yellow fever (Mapossa *et al*., 2021). Mosquito-borne diseases are responsible for almost 700 million cases and more than a million deaths each year and amount to an economic cost of US $12 billion per year (Chilakam, *et al*., 2023). Among methods for control of these diseases is the disruption mosquito-host contact for example by use of repellents. The use of repellents reduces vector host interaction, reduces bites and associated distress (Mapossa *et al*., 2021).
Unfortunately, most repellents used currently are of synthetic origin and have been associated with toxicities and several adverse effects. Prolonged exposure to pyrethroids has adverse effects on children's nervous system and causes neurobehavioural disorders (Pitzer *et al*., 2021). *N, N*-diethyl-m-toluamide (DEET) is toxic to human (Legeay *et al*., 2016; Briassoulis, *et al*., 2001), affects plastics and some fabrics and there are concerns as some mosquitoes have started developing resistance as has been expressed by service members of the US military, its largest number of users (Sanders *et al*. 2005).
Extensive use of chemicals for vector control results in environmental toxicities, non-acceptance by users and overall resistance by the insects (Shyamapada, 2011; van den Berg *et al*., 2012). This calls for search of safe and efficacious repellents and folklore is an important start point. Plants and plant products have been known and used as repellent and insecticides due to their large reservoir of bioactive substances and also because plant derived products are majorly low-cost and easy-access alternative (Santos *et al*., 2022). Plant derived botanicals do not leave residues on food, neither do they persist in the environment nor have major effects in organisms or plants that they are not intended to be used against. They can substitute synthetic insecticides or complement the use of the same (Ahmed *et al*., 2021; Souto *et al*., 2021).
Almost 2500 plant species have been identified to have pesticide activity. However, only a few of plant derived biopesticides have been developed, investigated for their possible activity or have reached commercial status in the recent past (Souto et al., 2021). The purpose of this study was to formulate a plant-based mosquito repellent that is effective and safe to use.

MATERIALS AND METHODS

The plants

Selection of the plants and collection of plant material

The plants for this study were selected on their ethnomedicinal information on their use for control of mosquitoes (Nguta *et al*., 2010). This was in addition to review of relevant literature on plants used for mosquito control. Six plants were identified for this study (Table 1). Plants were collected after initial field identification with the help of traditional herbal practitioners from Msambweni Sub County. Kwale county, Kenya. Further identification was done by a plant taxonomist at the Department of Land Resource Management and Agricultural Technology (LARMAT) of the University of Nairobi where voucher specimens were deposited.

To obtain a high-quality efficacious herbal drug, the appropriate part of the medicinal plant must be harvested at the optimum stage of development (Pandey and Savita, 2017). Harvesting of the plants' parts was done on the months of September and November when there is adequate foliage following the rains and material of best quality is ensured. The harvested plant parts were first cleaned with water then dried off the water and stored in dry sacks. The material was then transported to the Department of Public Health, Pharmacology and Toxicology, University of Nairobi where further preparation was done according to suggested procedures by Wagate, *et al*, (2008).

Utilization of plants in this study was based on their mosquito repellency activity. In comparison, acetone extracts had greater activity than the hexane and aqueous extracts as shown in table 2, Therefore, for the formulation of the biopesticide, acetone extracts of the plants' species were used.

Table 1: Plant species collected from Msambweni sub-county, Kwale county, Kenya

Family	Plant species, voucher specimen	Local name	Life form	Part used
Asteraceae	*Tagetus minuta* L. (JM 17)	Bangi ya shambani	Herb	Whole plant
Bombacacea	*Adansonia digitata* Linn. (JM 09)	Mbuyu / Mbamburi	Tree	Leaves
Labiatae	*Ocimum suave* Willd (JM 05)	Kirihani/Kivumbani	Herb	Whole plant
Labiatae	*Plectranthus barbatus* Andr.(JM 03)	Kizimwilo	Shrub	Leaves
Meliaceae	*Azadirachta indica* (L) Burm. (JM 10)	Mwarobaini/ Mkilifi	Tree	Leaves
Verbenaceae	*Lantana camara L* (JM 11)	Mjasasa	Shrub	Leaves

Table 2: Protection efficacy of extracts and controls

Plant	Acetone Mean±SE	Hexane Mean±SE	Aqueous Mean±SE	*P*-value
Lantana camara	98.33±1.67^{Bc}	94.86±0.53^{Bc}	77.63±1.04^{Ab}	<0.001
Tagetes minuta	98.33±1.67^{c}	98.33±1.67^{c}	94.86±0.53^{c}	0.182
Azadirachta indica	98.33±1.67^{c}	94.86±0.53^{c}	94.86±0.53^{c}	0.070
Adansonia digitata	84.58±1.58^{b}	75.27±4.07^{b}	80.00±4.71^{b}	0.259
Ocimum suave	100.00±0.00^{B}	94.86±0.53^{Ac}	94.86±0.53^{Ac}	<0.001
Plectranthus barbatus	84.47±2.70^{b}	74.14±3.59^{b}	80.53±2.81^{b}	0.107
DEET	98.33±1.67^{c}	98.33±1.67^{c}	98.33±1.67^{c}	-
Ethanol	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	-
P-value	<0.001	<0.001	<0.001	

Values of mean followed by similar letter/ capital letter(s) in the same row are not significantly different from one another (One –way ANOVA, SNK-test, α=0.05)

Formulation of the extracts

The test extracts were formulated in pure petroleum jelly. The formulations were made into concentrations of 10% and 20% of plant extracts in petroleum jelly. To make 10% of the formulation, fifty-four (54) grams of the pure petroleum jelly was weighed and transferred to a clean 100 ml beaker. The beaker with the pure petroleum jelly was warmed in a laboratory water bath at 80 °C and stirred with a stirring rod until it was fully melted. One gram (1 gm) of each of the six plant extracts was then added to the melted jelly and stirred continuously until it mixed fully with the petroleum jelly. Upon complete mixing, the resultant formulation was stored at $^{+}4^{o}$C awaiting repellency testing. To make 20% of the formulation, forty-eight (48) gram of pure petroleum jelly was melted in a beaker using water bath at 80 °C. Two grams (2 gm) of each of the six plant extracts added to the melted petroleum jelly and stirred continuously until full mixing. The resultant formulation was stored at $^{+}4^{o}$C awaiting acute dermal irritation/corrosion and repellency testing.

Acute dermal irritation test of the formulation

In assessment and evaluation of substances on human skin, the acute dermal irritation/corrosion test is necessary. This is done to determine the degree of irritation that a dilution of a test material can produce on the skin of New Zealand white rabbit, usually three per dilution of test substance (OECD, 20015). It provides information on absorption and possible risks including the mode of toxic action of a substance by topical route from short-term exposures through the skin. It is an initial part in determining a dosage regimen for subsequent studies (Chaudhary *et al*., 2008; Draize, 1965). To guarantee safety and care of the test animals for the acute dermal, the study protocol was submitted to and approval obtained from the Faculty of Veterinary Medicine Biosafety, Animal Use and Ethics Committee of the University of Nairobi.

The test animals

According to Jung *et al*, (2021), the New Zealand white rabbit is an appropriate model for this study since the results can be easily compared to other data bases and extrapolated to human. Three New Zealand white rabbits per plant extract per concentration were used. They weighed 2.5- 3 kg and aged 18-20 weeks. They were housed individually in the animal house at the Department of Public Health, Pharmacology and Toxicology of the University of Nairobi in relative humidity of 50-60% and lighting simulating day and night with conventional laboratory diet and unrestricted access to water (; OECD, 2015; Wang *et al*., 2017;).

The procedure for Acute dermal irritation testing

This was performed on intact and abraded skin of rabbits. Only animals with healthy intact epidermis by gross observation were used for the study, and three rabbits were used per test. For intact skin, prior to the test, fur was removed through shaving the left and right dorsal areas of the trunk of the animals. The skin was cleaned with distilled water and left for 24 hours. This was to allow for recovery of the *stratum corneum* from any disturbance caused by the shaving (Payasi *et al*., 2010). Testing on abraded skin was done to simulate situations when the skin has wounds, pimples or scratches. The same procedure as for the intact skin was used except that the shaved skin was rubbed with a fine abrasive paper (Amasa *et al*., 2012). Half a milliliter (0.5 ml) of each formulation was spread evenly to about 6 cm^2 of skin on the left dorsal area. It was covered with a gauze patch held in place by a non-irritating tape. The shaved skin on the right side applied with only 0.5 ml of distilled water was the control (OECD, 2015).

Trunks of the animals were wrapped with corsets to prevent them from interfering with the patches. After four (4) hours, the formulations were cleaned off by gentle swabbing with cotton wool soaked in distilled water. The animals were observed for signs of irritation such as erythema and oedema (OECD, 2015). Findings were scored at 1 hr, 24 hrs, 48 hrs and 72 hours after patch removal. The animals were further observed for any signs of dermatoxicity, behavior, general condition, posture and reflexes, attitude towards food, water, and hygiene on days 7 and 14 (OECD, 20015) They were also weighed on day 0 and the last day of the experiments.

Determination of Primary Irritation Index

The primary irritation index (PII):

PII = Σ (erythema grade at 24, 48 and 72 hr) + Σ (oedema grade at 24, 48 and 72 hr)/ total number of observations (Kapoor and Saraf, 2008).

Test mosquitoes

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

Cage tests

Cage tests were performed in 40 x 40 x 40 cm cages according to Innocent *et al*., (2010) with a 12:12 (Light: Dark) photoperiod and controlled temperatures of about 27^0C and a relative humidity of 80% maintained by use of an electric fan heater (WHO 1996, 2009). Active female host-seeking *A. aegyptiae* mosquitoes aged 3-7 days were collected from stock population using an aspirator and starved for the preceding 24 hours. Acetone/petroleum jelly mixture (1:1) was negative control while 20% DEET (*N*, *N*-diethyl-m-toluamide) was the positive control.

Volunteers who had avoided use of fragrance, any mosquito repellent, perfumed soap or tobacco for 12 hours prior and during the experiment (WHO, 2009; Innocent *et al*., 2010) were used for the experiments. The forearm, from the elbow to the wrist (~696.6 cm^2) was rinsed with water then dried in air. The extracts were applied as evenly and as thinly as possible. The rest of the hand from the wrist to the fingers was covered with latex glove to prevent the mosquitoes from biting (WHO, 2009). Permission for use of volunteers was obtained from Kenyatta national hospital and university of Nairobi ethical review committee (KNH/UoN ERC) under protocol number P357/05/2015.

Acetone/petroleum jelly mixture was applied on the other forearm that had been prepared as above, and served as negative control. The volunteer’s forearm that had been prepared as above was introduced into the cage through the sleeve for five (5) minutes. Mosquitoes that landed on or probed during this period were counted and shaken off before they can imbibe any blood (WHO, 2009; Innocent *et al*., 2010). Each trial was carried out in triplicate on different days and with different volunteers. The number of bites during the periods from 0 to 2 mins, 2–15 min, and 15–60 min were counted in order to estimate the protection percentage for each interval. Percent protection efficacy (PE) determined according to Ansari *et al*., (2005) & Phasomkusolsil and Soonwera (2010).

Percent protection efficacy (PE) = (C-T)/C x 100. Where C and T are the mean numbers of mosquitoes that landed on the control and test arm respectively.

Determination of knockdown effect of formulated product

Knockdown effect of the formulations was determined using WHO bioassay method (WHO, 2013). Tests were done in triplicates with positive and negative controls. Filter papers were treated with the formulated product and then air dried. Each was inserted into a chamber.

Twenty-five active *A. aegyptiae* mosquitoes aged 3-7 days that had not been blood-fed were selected using an aspirator from the stock populations of adult mosquitoes and used for this test. They were placed in each of the chamber with filter papers with different concentrations of formulated product for 1 hour.

Untreated filter papers were negative control while citronella oil at concentrations of 500 mg/m^2 was positive control. After one hour the mosquitoes were transferred to different holding chambers that had cups with 10% sucrose solution for the mosquito to feed. Mortality and recovery within 24 hours were scored and the time taken to knock down 90% of the population (KD_{90}) at 95% confidence interval was determined.

RESULTS

Acute dermal irritation/corrosion test

Fig1. Shows abraded skin of a rabbit healing well after application of the formulations. The results on both intact and abraded skin showed that the tested formulations did not cause any toxicity even after 14 days of observation. There were no signs of acute dermal toxicity such as redness, erythema, oedema or eschar. There was no significant change in weight of the test animals during the treatment period and there were no mortalities. Primary Irritation Index was zero for all treatments as the parameters for its determination were absent.

Fig 1. An abraded skin area of a rabbit and the rabbit skin healing well after application of the formulated products

Repellency testing

The two formulated products exhibited a repellency effect greater than or similar to DEET, the positive control as shown in tables 3 below. Product A which contained 10% of the plant extracts had repellent effect similar to DEET. Product B comprised of 20% of the plant extracts and offered 100% protection. This was greater than that was offered by DEET. The difference in activity of the formulated products and DEET was not statistically significant.

Table 3: Percent (%) protection of formulated products compared to DEET

Treatment	% Protection
Product A (10%)	98.33±1.67
Product B (20%)	100.00±0.00
Ethanol (Negative control)	0.00±0.00
DEET 20 %(Positive control)	98.33±1.67
P-value	<0.001

Knockdown effect

Fig. 2 and Table 4. shows the knockdown effects of the formulations. Table 4 shows that the knockdown effect of the products. Product A had a knockdown effect similar to citronella oil. Product B had greater knockdown effect compared to citronella oil even though the difference in activity was not statistically significant. In **Fig 2**, all the mosquitoes under study were immobilized in less than five minutes.

Table 4: Knock down effect of formulated products and citronella oil

Treatment	%Knockdown
Product A (10%)	93.33±3.33
Product B (20%)	96.67±3.33
Negative Control	13.33±3.33
Citronella oil	93.33±3.33
P-value	<0.001

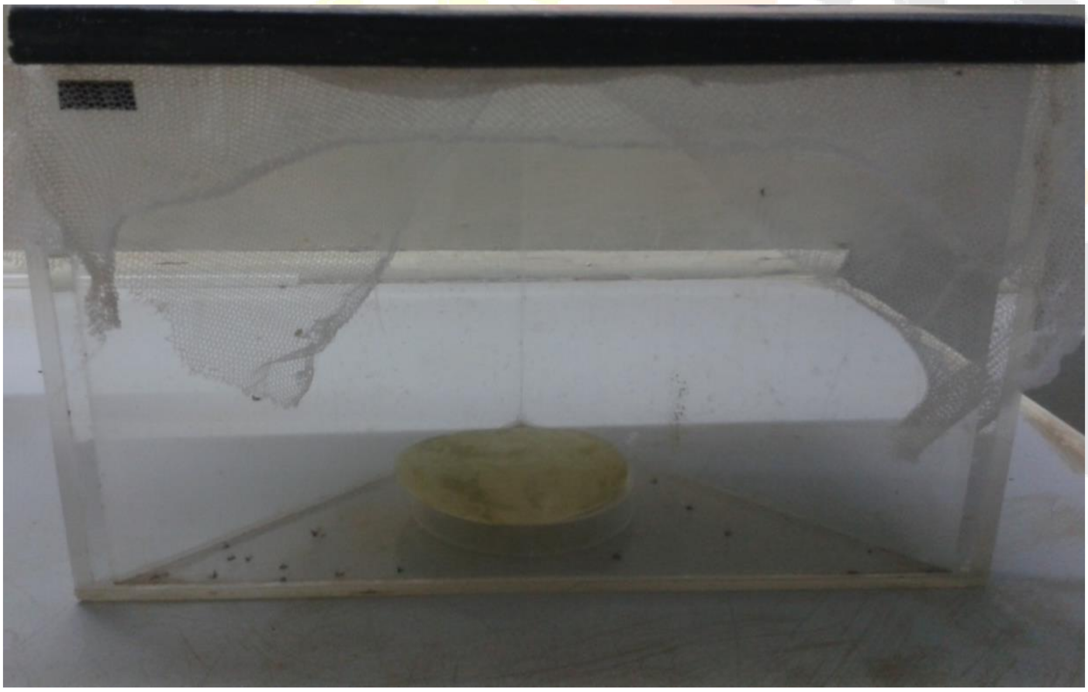

Fig 2: Mosquito knockdown effect of formulation A

All mosquitoes were dead/ immobile in the first three minutes. They never recovered even when they were transferred to the recovery chamber

DISCUSSION

Material to be applied on human skin should be assessed for irritability and corrosion potential. The acute dermal irritation/corrosion test is used because results obtained can be extrapolated to human (Sanders, 2007). It is useful in determining the mode of toxicity of a substance through the skin (Draize, 1965). The formulated products in this study were not irritating to both the intact and abraded skins of rabbits and human.

From the results, the formulated products had good repellency and knockdown effect suggesting there was synergism among the plant extracts. The knockdown effect of both formulations was equal or greater than that of citronella oil which was the positive control. Similarly, the percent protection offered by the each of the two formulations was equal or greater than that of DEET, the positive control. According to Logan *et al*., (2010), the drawback of using plant-derived repellents is that most consist of volatile substances making them only useful for short durations and requiring applications ever so often.

Formulation with petroleum jelly contributed to the improved activity as many researchers have also reported improved repellency after addition of a fixative substance to the repellents (Govindarajan, 2014, Kiplang'at and Mwangi, 2014). Besides 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 (Oyedele et al., 2002). The developed products can substitute the more expensive conventional repellents as they offer good protection against mosquitoes in the form of great repellent effect and knockdown activity and did not have adverse effects on the rabbit and human skins

CONCLUSIONS

From the results, the two formulated products were not irritating to the rabbit and human skin. They had excellent repellency and knockdown effect due to synergism among the plants' extracts. The formulated products can be used as mosquito repellent agents as alternatives to synthetic mosquito repellents. Formulating the products with a fixing agent, in this case the petroleum jelly, enhanced the activity of the formulations by preventing loss of active compounds as has been observed by other researchers.

RECOMMENDATIONS

Further research is needed to evaluate the activity of the formulated products in both semi field and field trials. Preservatives, stabilizers, antioxidants should be considered and the formulated products should also be assessed for shelf life and stability. The formulated products should be evaluated for commercial viability. Studies should be carried out concerning conservation of these plants and all the others that have been reported to have activities against different stages of mosquitoes.

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