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Mosquito Repellent and Mosquitocidal Effects of Neem (*Azadirachta indica*) on Human Beings in Imo State, Nigeria

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ABSTRACT

This study was aimed at determining the mosquito repellent and mosquitocidal effects of volatile oil of locally acclaimed plant Neem (Azadirachta indica), using human baits. Two different formulations, 30% ($^{v}/_{v}$) and 60% ($^{v}/_{v}$) were made and used on twelve human volunteers across three sampling locations (Ihiagwa, Royce and Obinze). The volatile oil was extracted using soxhlet apparatus, with petroleum ether as solvent. Olive oil was used as base for formulation and as a control. The topical application of each lotion reduced the biting rate of mosquitoes across the three locations, with the 60% (v/v) exhibiting higher impact of 341 vs 38; 426 vs 49 and 249 vs 45. 60% ($^{v}/_{v}$) formulation also had the highest percentage repellence of 88.86% at Location A and offered a whole night protection against mosquito in location O and offered a Complete Protection Time (CPT) of 17.7, 12.3 and 14.1 respectively across all three locations. There was a significant statistical association $r=0.95$ ($p<0.05$) between concentrations of the Neem oil formulations and mean duration protection time against mosquitoes. 60% ($^{v}/_{v}$) formulation had some mosquitocidal action by causing fast immobilization or paralyzing effect on some mosquitoes that were at close range to treated volunteer's body. The study concludes that volatile oils of Neem possess mosquito (A. gambiae and An. funestus) repellent effects, especially at higher concentrations and can be used to reduce human-mosquito contacts and hence mosquito-borne diseases and irritation caused by their bites.

Keywords: *Azadirachta indica; Formulations; Mosquitocidal; Neem; Repellent; Volatile oil*

INTRODUCTION

Mosquitoes are serious threat to public health, transmitting several dangerous diseases for over two billion people in the tropics (Nwakocha, 2007). It is

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estimated by the World Health Organization (WHO, 2009) that every year, up to 500 million people worldwide suffer malaria attacks, 90% of these being Africans, and that there are globally over 2 million deaths yearly, 90% of which are Africans (Salako, 2002). Diseases of mosquitoes are transmitted to human beings through bites only. Over the years, there has been no effective vaccine available for the control of these diseases, thus prevention of mosquito bites is one of the main strategies to control or minimize incidence of these diseases.

Fradin (1998) noted that protection against mosquito bites can be best achieved by avoiding infested habitats, wearing protective clothing and applying insect repellent. The use of repellent has been advocated. Insect repellents date back to ancient times, with the use of tars, smokes, plant oils and other modalities (Peterson and Coats, 2001). Insect repellents work by masking human scent, making it impossible for the mosquito to sense the carbon dioxide and lactic acid present in sweat in humans and act as attractive substances for mosquitoes. The use of insect repellants can provide practical and economical means of preventing mosquito-borne diseases. It is important not only for local people in disease risk areas especially in tropical countries, but also for travelers who are vulnerable to diseases spread by mosquito vectors when they visit and seek leisure away from their home countries (Aremu, Femi-Oyewo and Popoola, 2009).

Azadirchata indica (Neem) is an evergreen, fast growing tree normally 15-20 metres in height in the mahogany family Meliaceae. They are widespread in tropical and subtropical regions of the world, including semiarid and wet-tropical regions (National Research Council, 2002). It is one of three species in the genus *Azadirchata*, and is native to Indian subcontinent, growing in tropical and semitropical regions (Niyi, 2011). It is widely available in the developing world, and known by different names such as Nim (Bengali), Tamar (Burmese) and Grossblacttigerzedrach (German). In Swahili, it is called Muarubaini which means the tree of the 40, as it is said to treat 40 different diseases. In northern Nigeria, it is called *Dongoyaro* (literarily; “boy standing tall”) to describe the healthy appearance of those treated by the herb. In Sanskrit, Neem is known as Arishta, which translates as “reliever of sickness” (Niyi, 2011).

In India, Neem is known as “the village pharmacy” because of its healing versatility, and it has been used in Ayurvedic medicine for more than 4,000 years due to its medicinal properties (Singhal and Mudgal, 1983). Neem seeds contain approximately 99 biologically active compounds of which azadirachtin, nimbin, nimbidin and nimbolides are major molecules. Many of these derived products have anti-feedancy, ovicidal activity, fecundity suppression besides insect growth regulation and repellency against insects (Schmutterer, 2002; Isman 2006). The seeds bark and leaves contain compounds with proven antiseptic, antiviral,

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antipyretic, anti-inflammatory, anti-ulcer, anti-fungal properties (Sudhir *et al*., 2010). Essential oils are volatile naturally occurring, complex compounds characterized by a strong odour and are formed by plants as secondary metabolites (Patel, Gupta and Oswal, 2012). Neem oil is yellow to brown in colour, it has a bitter taste, and a garlic/sulfur smell. It has been used for hundreds of years to control pests and diseases. It is composed mainly of triglycerides and contains many tri-terpenoid compounds, which are responsible for the bitter taste. The active ingredients include: Azadirachtin, Nimbin, Nimbidin, Nimbidol, Sodium nimbinate, Gedunin, Salannin, Quercetin (Sudhir *et al*., 2010). This study is aimed at determining the mosquito repellent and moquitocidal effects of the volatile oil of Neem.

MATERIALS AND METHOD

Study Design: This is a field trial experimental study that seeks to examine locally acclaimed plant that grows in Imo State, South East Nigeria, for mosquito repellence and mosquitocidal activities. Volatile oil were extracted from the leaves of the test plants formulated into two different concentrations (two formulations) and tested on volunteered human subjects. The study was conducted between September through October, 2017, using the extracted essential oils as test material and olive oil as negative control.

Description of Study Area: This study was conducted in Owerri. Owerri is made up of three local government areas, which are Owerri West, Owerri North and Owerri Municipal. All these places lie within the rainforest zone of the south-eastern Nigeria with typical tropical climate. The temperature range during the study period was 35-40°C. Owerri has a tropical wet climate according to the Köppen-Geiger system. Rain falls for most months of the year with a brief dry season. The study was carried out in three different locations Ihiagwa, Royce Road and Obinze.

Study Population: The study population for this study comprise individuals living in the three locations, which are Owerri West, Owerri North and Owerri Municipal. Selection of houses to be included in the investigation was done by the researcher. Selection criteria included the presence of stagnant water collections in gutters and potholes as well as refuse dumps littering the vicinity of the houses. Such practices promote proliferation of mosquitoes. Houses where mosquitoes were found inside bathroom floors and around water closets and septic tanks were enlisted.

Sample Size: In each of the three locations, four (4) human baits who voluntarily

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consented were selected and were used. Since it has been established that only about 20% in a population receive intensive bites from mosquitoes due to chemical makeup and other factors (Du, 2005), the investigator selected only those who claim to experience much mosquito bites naturally. With the help of the University Director of Health Services, the subjects were given chemo prophylactics before the investigation and they continued it throughout the period of investigation and two weeks after the exercise. Thus, the sampling size for this study is 12.

Sample Preparation: Fresh leaves of *A. indica* were collected along the back gate of Federal University of Technology Owerri. The leaves were air dried until the moisture content was reduced to barest minimum. The collected leaves of each of the plant materials were pulverized into powder using a Binatone Mx10 blender and sieved to obtain a fine powder of the plant part. 150g of each pulverised plant part was placed in a plain sheet of white paper, then placed in the timbel of the soxhlet apparatus compartment using petroleum ether extraction solvent (Okigbo *et al*, 2010). The oils extracted were stored in an appropriate sample bottles at a temperature of 4^0C till the period of formulation and repellency testing.

Formulation of Oil: Olive oil was used as the natural oil base for the formulations. Two different concentrations (30% $^{v}/_{v}$, and 60% $^{v}/_{v}$,) were made for the essential oil. The concentrations were made following the method employed by earlier researchers (Oyedele, *et al*., 2000; Oparaocha, *et al*., 2010). The olive oil was bought from Priceless Supermarket in Owerri. The different formulations of Neem oil were labeled A_1 and A_2. The ordinary olive oil which served as negative control was labeled CONTROL.

Mosquito Repellent Test Procedure: The method adopted for mosquito repellency test is that developed by Oparaocha *et al*, (2010) but was adapted using WHO (2009) procedures for field trial. The volunteers worked in pairs and for each of the six in each location, a volunteer used the negative Control. All the six pairs worked for three sessions (morning, evening and night). The pairs for the three sessions worked for three consecutive days, twice a month and rotated lotion type and session every day. All the formulations were tested the same day so as to allow equal exposure to the same environmental conditions. For the testing, 2ml of the test lotion was given to the volunteers to rub on the exposed parts of the body (the arms, legs and on the face). The pairs that worked in the morning and evening sessions stayed outside the house at about 20 m distance from each other from 5:30am to 7:30am and from 6:00pm to 8:00pm, while the pair for the night stayed in different rooms in the same house. The volunteers were informed to note the number of flies resting on the exposed body parts by

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catching them with inverted cylinder tube. For those working at night, since they were not expected to get up from bed to catch mosquitoes, a grading system was introduced to them to record palpable bites and/or number of times they were woken up by the buzzing sound of mosquitoes as below; 0 bites/buzzing sound (no bite), 1–2 bites/buzzing sound at the same time and woken up to three times (mild bite), 3 and above bites at the same time and woken up more than three times (heavy bites). Total bites for each month (24 h) of morning and evening as well as the two-month totals were calculated for each lotion type for the treated and control. For whole night bites a summary of their reports was made. A randomized species identification of the mosquitoes was done by an entomologist.

Mosquitocidal Test Procedure: The method utilized was developed by Oparaocha *et* al, (2010). It is a very simple method, in which the volunteers were told to note any dead mosquito or any immobilizing effect of the lotion on the fly. The investigator confirmed the action before recording.

Ethical clearance: Ethical clearance was obtained from the Department of Public Health before commencing the investigation. Informed and free consents were obtained from the volunteers before they were recruited for the study.

Data Analysis: The repellence for the treated and the control in each test location was recorded and the relationship between concentrations of repellence of formulations and mean protection time was measured using Pearson's correlation coefficient. Complete Protection Time (CPT) and Percentage Repellency were calculated for each of the formulations. The results were presented in tables and charts. Biting rate is an important parameter for assessing malaria transmission and evaluating vector control interventions, is commonly estimated by Human Landing Collections (HLC) (Kwon *et al*.., 2011). Percentage Repellency is the number of mosquitoes landing or attempting to bite at given time intervals without re-treatment is recorded and the repellence of the substance is calculated with the formula:

$$\% \text{ Protection} = \frac{C-T}{C} \times 100\%$$

Where C corresponds to the number of bites by exposing the control limb (untreated or with a solvent such as Olive oil as a control substance) and T by exposing the repellent treated limb. Complete Protection Time (CPT) is the time period between repellent application and the first two mosquito landing/probing/ bites during the same observation period or one bite each in two consecutive intervals.

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RESULTS AND DISCUSSION

Repellence Result: Biting Rate: The results of monthly biting rates of mosquitoes on both treated and control test volunteers at different concentrations and at different locations is shown in Table 1. It can be seen from the results that there is significant reduction in the biting rate for the two different formulations at the three locations (Ihiagwa, Obinze and Royce Road), with the 60% ($^{v}/_{v}$) formulation exhibiting highest impact (341 *vs* 38; 426 *vs* 49 and 249 *vs* 45) at all the centres respectively, for the two months totals, while the 30% ($^{v}/_{v}$) formulation had the least impact (357 *vs* 47; 382 *vs* 49 and 262 *vs* 41), respectively. The highest biting rate (426 *vs* 49) was recorded at Royce.

Percentage Repellence: The results of the percentage repellence showed values ranging from 74.77 to 91.47% for the two-month averages, with the 60% ($^{v}/_{v}$) formulation having the highest values (88.86, 88.50 and 85.27%), while the 30% ($^{v}/_{v}$) formulation had the least (86.83, 87.17 and 81.03%) in Ihiagwa, Obinze and Royce road (Table 2).

Complete Protection Time (CPT): The result of the complete protection time for the 24 hours/2-months biting time is given in table 3 below. It can be seen that across the three test locations, the 60% ($^{v}/_{v}$) formulation offered a CPT of 17.7, 12.3 and 14.1 respectively across the three locations. Similarly, the 30% ($^{v}/_{v}$) formulation offered a CPT of 14.1, 16.8 and 17.1 respectively across the three locations

Whole Night Biting Rate: The result of the whole night protection offered by the different formulations of repellents is shown in table 4 below. From the table it can be seen that all the formulations offered a significant reduction in biting rate, with the 60% ($^{v}/_{v}$) formulation offering whole night protection against mosquito bites in Obinze. However, mild bite were reported across Ihiagwa and Royce.

Correlation between Concentration of Neem Oil and Mean Protection Time

$r = \{[\sum(x-x)(y-y)] / \sqrt{\sum(x-x)^2 (y-y)^2}\}$,

Where 'r' is correlation coefficient

$\sum x = 22.06.0$, $\sum y = 12.18$, $\sum xy = 104.73$, $\sum x \sum y = 72.1$

Therefore, $r = 32.63 / 34.24 = 0.95$

Value of correlation coefficient' r' indicates that there is close association between concentrations of the Neem oil formulations and mean duration protection time against mosquitoes. To test whether the observed correlation is due to chance or not, a student t- test is used to determine the significance at 5% level

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$t = \{r\sqrt{[(n-2)/(1-r^2)}\}$, df = n-2
$t = \{0.95\sqrt{[3/(1-0.903)]}\} = 5.29$, df = 3

This is significant at 5% level, confirming the significance of the apparent association between concentrations of the formulations cream and mean duration protection time.

Mosquitocidal Effects: The mosquitocidal effects of the 2 formulations were also observed. All the formulations showed varying levels of paralyzing and knock down effects. Observation showed that the 60% formulations had the highest and higher mosquitocidal action across the three test locations, by causing fast immobilization or paralyzing effect on some mosquitoes that were at close range to treated volunteer's body. The affected mosquitoes were immobilized and can easily fall off the volunteers hand to the ground. In all, only six mosquitoes demonstrated this fast knockdown effect of the volatile oil during the two-month study and the observation was made only in Royce. A random specie identification show that 81.7% of the collected mosquitoes were *A. gambiae* and 18.3% were *An. funestus.*

Adverse Reaction Enquiry Result: The volunteers were also asked on the occurrence of any skin reaction as a result of the topical application of the repellents. None of the volunteers reported any adverse reaction on the body parts to which the repellents were applied (arm, leg and face) across the three test locations. This was also confirmed by a preliminary physical observatory test carried out on the volunteers by a dermatologist. However, volunteers reported high sensation of warmth (heat) as a result of application of the test lotions.

Repellents are substances that act locally or at a distance, deterring an arthropod (insect/mosquito) from flying to, landing on or biting human or animal skin (Sah *et al*., 2010). Volatile oils of *Azadirechta indica* have shown from this study, to possess mosquito repelling abilities. The result showed that all the two types of repellent formulations offered a considerable amount of protection from mosquito species. This has corroborated the results of previous studies (Mordue and Nisbet, 2000; Abdelkarim & Heinz, 2006; Aremu, Femi-Oyewo & Popoola, 2009). Sharma, Ansari and Razdan (1993) proved that two percent *Azadirechta indica* oil mixed in coconut oil, when applied to the exposed body parts of human volunteers, provided complete protection for 12 h from the bites of all anopheline species. In another study, Sharma (1995), evaluated repellent action of Neem oil against different mosquito species. Two (2) % Neem oil mixed in coconut oil provided 96-100% protection from *Anopheline*s, 85% from Aedes, 37.5% from *Armigeres*, whereas it showed wide range of efficacy from 61-94% against *Culex* spp.

This study shows that at higher concentrations, *Azadirechta indica* oil offer higher protection from mosquito species. This was evident from the correlation coefficient' r' that indicated that there is significant statistical association r=0.95 ($p<0.05$) between concentrations of the Neem oil formulations and mean duration protection time against mosquitoes. Thus, it is not by chance. This is in conformity with previous studies. Jilani *et al.* (1988) tried the repellent and growth-inhibiting effects of turmeric oil (*Curcuma longa*), sweetflag oil (*Acorus calamus*), Neem oil (*Azadirachta indica*), and Margosan-O (a commercial Neem-based insecticide) on red flour beetles (Tenebrionidae). It was found that the repellence increased with increasing concentration of the oils and Margosan-O, while the turmeric oil or sweetflag oil repelled insects during the first 2 weeks, while some essential oils have been used as insect repellents since ancient ages such as citronella and ennyroyal.

In a field study in India by Sharma *et al* (1995), it was reported that direct burning of Neem leaves offer 76.0% protection from mosquitoes for 2 hours. Field study in India by Ansari, Mittal, Razdan and Sreehari (2005) with 1% neem oil volatilized in a kerosene lamp offer 94.2% protection from *Anopheles spp*. and 80% protection from *Culex spp*. The results also indicated that the test oil afforded whole night and to a greater extent, dawn and dusk protection at 30% (v/v) concentration in the olive oil base. Malaria mosquitoes bite mainly during night, dusk and dawn.

Sharma and Dhiman, (1993), in another study proved the repellent action of Neem oil against sand flies under laboratory and field conditions. Concentrations of 2% Neem oil mixed in coconut or mustard oil provided 100% protection against *Phlebotomus argentipes* throughout the night under field conditions; against *Phlebotomus papatasi* it repelled for about 7 h in the laboratory. Same was observed by Sharma *et al.* (1993), when they evaluated repellent action of Neem oil against different mosquito species. They found out that 2% Neem oil mixed in coconut oil provided 96-100% protection from a*nopheline*s, 85% from Aedes, 37.5% from *Armigeres* whereas it showed wide range of efficacy from 61-94% against *Culex* spp. Observation on the mosquitocidal impact of the formulations indicated that the 60% ($^{v}/_{v}$) formulation had some mosquitocidal action by causing fast immobilization or paralyzing effect on some mosquitoes that were at close range to treated volunteer's body.

The affected mosquitoes were immobilized and can easily fall off the volunteers hand to the ground. The mosquitocidal effects could be due to the presence of Nimocinol in Neem leaves, which possess insecticidal effects (Atawodi and Atawodi, 2009). Ebe *et al*, (2015), in their study "Comparative Analysis of Petroleum Ether and Aqueous Extracts of Neem Leaf and Neem

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Stem on Different Stages of *Anopheles gambiae*", observed that the petroleum ether extract of Neem oil has adulticidal effects on the mosquitoe specie. The seed kernel of *Azadirachta indica*, the leaf of *Cymbopogon nardus* and the wood of *Fernandoa adenophylla* have also been reported to have adulticidal properties against *Aedes aegypti* (Zaridah, Nor and Rohani, 2006).

A preliminary dermatological study on the effects from the topical application of all the repellent formulations and oral interview of the volunteers for the study showed that there were no adverse impact in any form on their skins during the two-month study. Documented evidence has shown that repellents of plant origin do not pose hazards of toxicity to humans and domestic animals and are easily biodegradable. Natural products are safe for humans when compared to that of synthetic compounds (Sharma *et al*., 1993; Mittal and Subbaro, 2003).

CONCLUSION

This study can conclude that volatile oils of *Azadirachta indica* possess mosquito (*A. gambiae* and *An. funestus*) repellent effects, especially at higher concentrations. It has also been established from this study that effective protection offered by the repellent is dependent on concentration of the repellent formulation. This study also concludes that volatile oils of *Azadirachta indica* possess mosquitocidal effects, in which mosquitoes are immobilized and paralyzed on contact with a skin treated with the formulation. Malaria mosquitoes bite mainly during night, dusk and dawn. Consequently, the oil of *Azadirachta indica* could be used as an alternative, relatively safe, natural, insect-repellent to protect people from mosquito bites at night when they are on bed, and at dawn and dusk when often due to poor housing and harsh climatic conditions as well as social and religious responsibilities stay outside their houses.

Since the extraction technique is simple, the colour, fragrance and consistency of the lotions remained unchanged during the two months study, and the oil base (olive oil) is readily available with a religious acclaimed benefit, the local people could be taught how to prepare the lotions. The formulation could equally help to supplement the protection afforded by window and door nets for those who because of cost and/or odour of permethrin do not like to sleep under insecticide-treated bed nets

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Table 1: Monthly and average mosquito biting rate (24hrs/month) on treated and control volunteers

Formulation/Month	Ihiagwa		Royce Road		Obinze	
	Treated	Control	Treated	Control	Treated	Control
30% ($^{v}/_{v}$) of *A. indica* in olive oil (A_1)						
September	18	211	23	237	21	146
October	29	146	26	145	27	107
Total	47	357	49	382	48	253
60% ($^{v}/_{v}$) of *A. indica* in olive oil (A_2)						
September	21	176	27	218	18	142
October	17	165	22	208	20	116
Total	38	341	49	426	38	258

Table 2: Monthly percentage repellents of the formulations in the three locations

Formulation/Month	Percentage repellent in the three locations		
	Ihiagwa	Royce Road	Obinze
30% ($^{v}/_{v}$) of *A. indica* in olive oil (A_1)			
September	91.47	90.30	85.62
October	80.14	82.07	74.77
Total	86.83	87.17	81.03
60% ($^{v}/_{v}$) of *A. indica* in olive oil (A_2)			
September	88.07	87.62	87.32
October	89.70	89.42	82.76
Total	88.86	88.50	85.27

Table 3: Complete Protection Time for 24hrs/month testing for September

Lotion type	Ihiagwa CPT	Royce Road CPT	Obinze CPT
Formulation A_1	14.1	12.3	14.1
Formulation A_2	17.7	16.8	17.1

Table 4: Whole night biting rates for 24hrs/month test duration

Lotion type	Ihiagwa		Royce Road		Obinze	
	T	C	T	C	T	C
Formulation A_1	+	++	+	++	+	++
Formulation A_2	+	++	+	++	0	++

Key: T=Treated, C=control, 0=No bite, +=Mild bite, ++=Heavy bite

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this study.

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