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Culicidae Repellency Property of Neem Seed Kernel Extract

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ABSTRACT The extract from the seed of the Neem tree (Azadirachta indica) is known for its use by traditional medicine practitioners and its local use as mosquito repellent but little is known about its chemical properties. This research studies the efficacy of the use of the Neem seed kernel extract (NSKE) as a mosquito (culicidae) repellent. Mosquito repellent is necessary considering that mosquitoes are detrimental to human health and economy. Local production and use of this repellent could enhance the Nigerian content in Phyto- chemical production and also increase the Nigerian gross domestic product. NSKE mosquito repellent is better than synthetic mosquito repellent for its low toxicity, high availability, stability, unexpiring characteristic, low production cost and ease of application. Activity test revealed that the repellent is active a short distance from its point of application and acts over a reasonable time interval. The repellent breaks away into harmless products within a reasonable time. By this, it cannot produce harmful effects on man nor pollute his environment (soil, water and air). Chromatographic and spectrophotometric analysis revealed that NSKE contains four methyl esters with 11, 14 – Eicosadienoic acid being the most active in the vapour phase. These were found to produce phago- repellent antifeeding effect but majority of the mosquitoes still recovered after the active period when the repellent was completely metabolized. This was okay, as the real effect is to stop the mosquitoes from biting not necessarily killing the mosquitoes. Insects have general behavioral characteristics; hence the product can also be used to repel insect pest such as: weevils, cockroaches, houseflies, etc. The repellent should be reapplied after the recommended active period.

Keywords: Phago-repellent, Active Period, Active area, Active component, Spectrophotometric, Culicidae, Neem Seed Kernel Extract

Introduction

Sampson (1999) state that although much work has been done on the production of insect repellent using petrochemicals, little has so far been done on the production of insect's repellent from a natural source which has no acute toxicity to mammals but with negligible or no chronic effects. Sampson (1999) empirically found that the NSKE is not lethal, it repels the target mosquitoes without causing a total irreversible unresponsiveness to tactile stimulation. According to world health organization (WHO) "malaria takes life every fifteen seconds" it kills more than AIDS, Cancer or Asthma (Kristof,1997). Most seeds, farm animals and domestic animal kept indoors cannot be attacked by insect pests where the repellent is burnt or sprayed. This study

is therefore of tremendous importance not only for improving the health of man but also for enhancing better storage not prone to attack by insect pest. Animal disease can also be prevented using this product.

Moreover, the psychological state of man is improved when he is in the best state of health. According to World Health Organization (WHO) this has a relationship to intellectuality (World health report,2001). It is therefore believed that if these disease vectors prevalent in the tropical region are gotten rid of or controlled effectively, the tropical man can easily measure up with the temperate men in terms of intellectuality. The result of this will be a fast-developed technology, which of course we are yearning for. Besides an improved technological development, there will also be an improvement in the efficiency of the labour force. The workers work faster and more accurately under the best health conditions. Having examined the importance of the product, it has been realized that the product is low cost, so it is possible to produce it in commercial scale to meet the demands of Nigerians for the products. A chemical process plant can therefore be established to produce the product in large capacity.

The natural extract of neem seed kernel is phago-repellent (Buckingham & Cadogan,1982). The insect may starve to death while remaining on its host in the presence of an anti-feeding compound (Green *etal*., 1987). Moreover, the larvae which do consume the compound despite the repellency have both molting and growth delayed and eventually die. The repellent is very effective a short distance from its point of application, hence more effective for indoor pests. According to food and agricultural organization (FAO) and world health organization (WHO) insecticides account for 500,000 poisonings and 500 deaths which occur annually in developing countries and this is a problem which the developing countries should address (WHO, 2014).

Insect Repellency Component

NSKE contains a natural insect pest repellent called Azadirachtin, a methyl ester and a powerful antifeedant with empirical formula $C_{35}H_{44}O_{16}$. It is built up of 17 memeberd, 36 membered and 25 membered rings which can be represented in planar form but with two ringed bridge. Four of the oxygen atoms are ring members but there is only one double bond. Three carboxyl groups are attached, two esterified with methyl and one with enol form of butanone. Two hydroxyl and one acetyloxyl groups are attached. Simpler synthetic compounds related to this complex structure are being sought. Azadirachtin ($C_{35}H_{44}O_{16}$) is a constituent of the seeds of melia Azadirachta and the Indian neem tree (Azadirachta indica). Buckingham&Cadogan (1982) states that Azadirachtin is a very active phago- repellent and systematic disruptor. The repellent is effective over a reasonable time interval and acts over a short distance from the point of its application. It acts by displacement or morbidity of the insect pest but discontinuation of application after a short period of activity results in re-occurrence of the insect pests. Throughout the active period of the repellent the target pest is rendered inactive. This means that there will be no mosquito bites during the active period.

Materials and Methods

Materials

The following materials were used for the study:

- Ati Matson infra- red Spectrophotometer; Schimadzu 160 A Ultra-Violet
- Spectrophotometer; Carbon-13 Nuclear Magnetic Resonance
- Spectrophotometer; Proton Nuclear Magnetic Resonance
- Spectrophotometer; Schimadzu Gas Chromatograph-Mass
- Spectrophotometer; Round bottomed flask; Anti-bumping granules; Neem
- Seed kernel extract; Petroleum ether; Sodium ethoxide; Methanol; Sodium hydrogen sulphate; Sodium sulphate.

Methods

Determination of Mosquito Repellency Component

Table 1: Spectrophotometric Methods of Analysis

Method	Application
UV&IR	Detection of functional groups
NMR	Detection of position of functional groups on parent atom
GC-MS	Detection of major components of interest in a sample

Infra-red Spectrophotometry

The neem seed kernel extract was bombarded with infra – red radiation. The wave band corresponding to different functional groups were used to detect the functional groups

Ultra -violet Spectrophotometry

The ultra-violet spectrophotometer was used to confirm the functional groups detected by the infra-red spectrophotometry and to differentiate between them.

Nuclear Magnetic Resonance

Besides proton NMR, the carbon-13 NMR spectrophotometer was used for this analysis because it allows more detailed analysis of structural features of fairly large molecules. The extract was placed in a magnetic field and bombarded with electromagnetic radiation. The orientation of the magnetic nuclei changed as a result of resonance absorption of electromagnetic radiation. From the NMR spectrum, the intensity of each peak was obtained as the area under each peak in the NMR spectrum. The position and intensity of lines in the NMR spectrum was used to locate the position of the functional groups as they are attached to the parent atom.

Gas Chromatograhpy-Mass Spectrophotometry

Methylation: Methylation was carried out in order to make the extract volatile enough not to block the chromatographic column and damage the equipment. 5 ml of the sample oil was added to 10 ml of petroleum ether in a round bottomed flask. A few drops of anti-bumping granules were added after which 1 ml of sodium ethoxide and 0.5N dry methanol was added. The mixture was refluxed for 5 minutes at 40 ^{0}C in a water bath and 2 ml of $NaHSO_4$ added in order to neutralize excess sodium ethoxide. The mixture was allowed to settle and the upper layer taken. A little Na_2SO_4 was then added in order to absorb moisture.

Equipment: The (GC-MS), a standard equipment, was used to detect the various components of the neem seed kernel extract. It has an inbuilt standard so it can detect even the minutest components very efficiently. The interfacing of chromatographic columns to mass spectrophotometers permits instantaneous display of the spectrum of each species as it leaves the chromatographic column. The instrument is also interfaced with a computer so that each spectrum is digitalized and stored for later production. With a gas chromatograph alone, components with almost similar peak heights and retention times may be imbibed in the other components, hence the need to combine gas chromatograph with mass spectrophotometer. A Mass spectrophotometer bombards the substance under investigation with an electron beam and quantitatively records the result as a spectrum of positive fragments.

Procedure: 1 μL of the methylated extract was weighed and injected into a carrier (He) gas supplied from high-pressure cylinders at constant flow rate and pressure. The injected sample was heated to vapour and carried by the carrier gas to a hydrogen flame ionization detector maintained at constant temperature and supplied with combustion air and hydrogen. The detector retention time (time taken to elude the column) and a recorder recorded peak heights of each component. The retention time was used to identify the component but the peak height was used to determine the amount of each component.

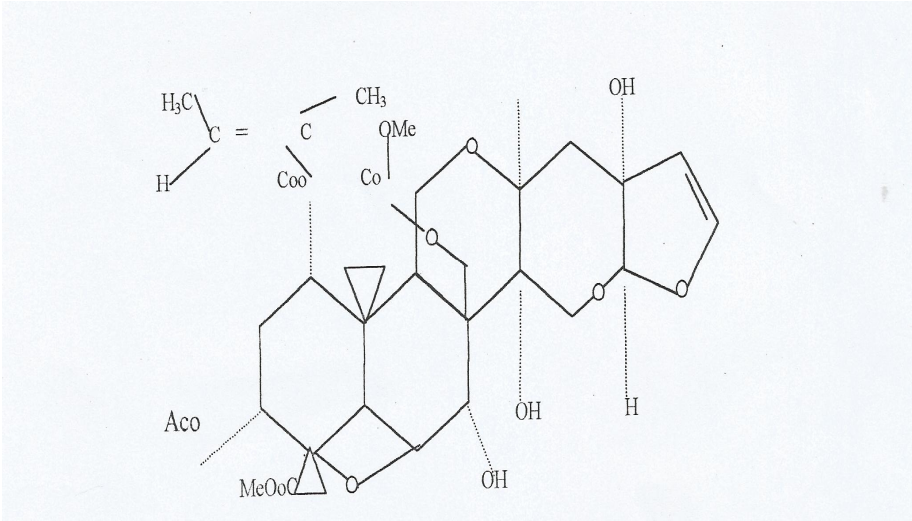
Results

UV and IR Spectrophotometry

The following Functional groups were detected:
CH_3; C = C; –COO; CO_2ME; AOC; –OH; –O; C-C; C-H

Nuclear Magnetic Resonance

The functional group detected by the ultraviolet (UV) and the infrared (I.R) spectrophotometry are attached at the following positions on the parent atom:



Chemical formala: $C_{35}H_4O_{16}$

Fig.1: Positions of Functional Groups on Parent Atom as detected by NMR

Gas Chromatography-Mass Spectrophotometry

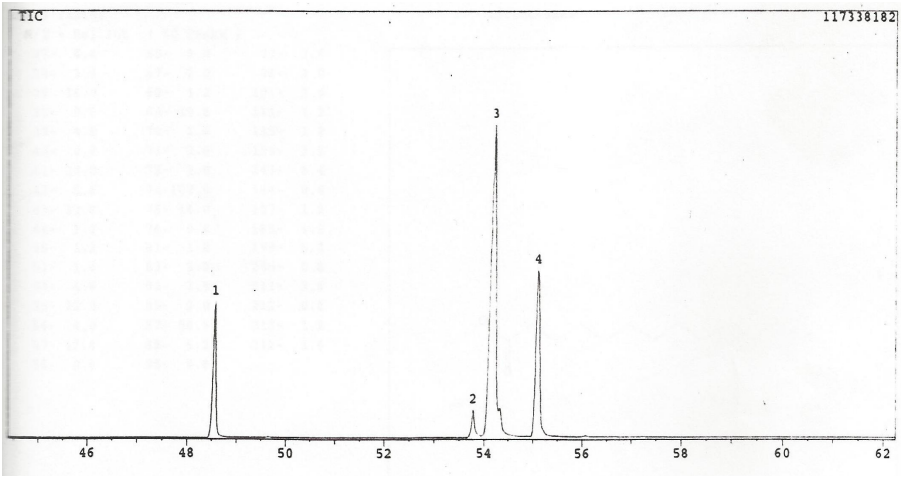


Fig 2: Gas Chromatography-Mass Spectrophotometry Waveform

From the GC –MS analysis and GC-MS library information the major components of the neem seed kernel extract were as follows:

Peak 1: Tetradecanoic Acid (Methly Ester)

Structure:

O

$CH_3(CH_2)_{12}$ C –OCH_3
Mol. Wt. 242 **Percentage**: 15.02 **Formula**: $C_{15}H_{30}O_2$
Peak 2: 11,14 –Eicosadienoic Acid (Methyl Ester)
Structure:

O

$CH_3(CH_2)_4$ CH = CH-CH_2 = CH – $(CH_2)_9$ C –OCH_3
Mol.Wt. 322 **Percentage:** 2.95 **Formula**: C_{21} H_{38} O_2
Peak 3: 10 – Octadecenoic Acid (Methyl Ester)
Structure

O

$CH_3(CH_2)_6CH$ =CH $(CH_2)_8$ – C – OCH_3
Mol.Wt. 296 **Percentage**:59.10 **Formula:** $C_{19}H_{36}O_2$
Peak 4: Octadecanoic Acid, (Methyl. Ester) or Stearic Acid
Structure

O

CH_3 $(CH_2)_{16}$ C –OCH_3
Mol.Wt. 298 **Percentage:** 22.92 **Formula**:$C_{19}H_{38}O_2$
Combustion Products
These methyl esters detected by GC-MS react with indigenous groups and Oxygen to produce toxic metabolites.

Formulation

Being considered to be the most reactive 11, 14 – Eicosadienoic acid is considered the active component for this formulation.
Basis: 0.5 ml oil + l ml kerosene = 1.5 ml (1)

Table 2: Formulation of the Repellent

Component	Calculation	Percentage
Kerosene	$\frac{1}{15} \times \frac{100}{1} = 66.6667$	66.6667
Eicosadienoic acid	$100 - 66.6667 = 33.33$ $\frac{2.95}{100} \times 33.33 = 0.9832\%$	0.9832
Other Components	100 – 66.6667 – 0.9832 = 32.3501%	32.3501
	Total	100

Applicability Characteristics

Subjecting a sample of the NSKE to extremes of temperature 0^0C, 50^0C and 100^0C, vibration, compression and to sunlight did not change the physical properties of the NSKE. Igniting a sample of the NSKE did not result in fire explosion.

Active Period

Table 3: Active Period for different methods of Application

Method of Application	Active Period
Burning	**As long as the Lantern keeps burning**
Rubbing	3 hours
Spraying	45 minutes

Active Area

Active area under closed door = Area of the room. (2)

Active area under open door = πr^2 (3)

3.142×50^2 cm = $3.142 \times 2500 = 7855$ cm^2 = 0.78855m^2

The number of lanterns needed per 0.7855m^2 = $\frac{\text{Area of the living room in m}^2}{0.7855\ \text{m}^2}$ (4)

If the room is square or rectangle or triangular in shape, the area is calculated in terms of a circle circumscribing the room.

Discussion

According to Basler (1991) a peak range of 425 -445 Nm show the presence of methyl ester. 441.0 Nm confirm methyl ester group. Figure 1 show the structure of Azadirachtin represented in planar form but with two ringed bridge. Four of the oxygen atoms are ring members but there is only one double bond. Three carboxyl groups are attached, two esterified with methyl and one with enol form of butanone. Two hydroxyl and one acetyloxyl groups are attached. Figure 2 show the wave form from the GC-MS equipment. This show that Azadirachtin transforms itself to four methyl esters among which 1, 4 - Eicosadienoic acid is the most active because of the presence of two double bonds. This is followed by 10 – Octadecenoic Acid which has only one double bond. Removal of one methyl group from each of the four methyl esters yield their corresponding free fatty acids which are palmitic. Arachidonic and oleic acids respectively, the fourth is stearic acid. According to Kenneth (1990) an insect repellent must have the following properties: A methoxyl group, Ester linkage to a leaving group, hydrophobicity and unsaturation. Another property that gives these methyl esters insect repellency property is that they are highly volatile, easily distilled and vaporized into air and hence effective in repelling the target mosquitoes. It is known that the presence of double bond shows the level of unsaturation and hence reactivity, therefore among the four methyl esters 11, 14 – Eicosadienoic

Acid appears to be the most active followed by 10-Octadecenoic Acid. Chromatographic and spectrophotometric analysis reveals that the neem seed kernel extract contains four methyl esters close to the structure of azadirachtin. These methyl esters are active, easily distilled and vaporized into air; hence their effectiveness in causing mosquito repellency action of the extract. 11, 14 – Eicosadienoic Acid is the most active because of the presence of two double bonds. These methyl esters detected by GC-MS react with indigenous groups and oxygen to produce toxic metabolites. That is why it is more effective when burnt. The combustion reaction increases the toxicity of the repellent to the target mosquitoes. A further research to investigate the combustion products may require Air space chromatography which is not available in Nigeria. Subjecting a sample of the NSKE to extremes of temperature 0^0C, 50^0C and 100^0C, vibration, compression and to sunlight without the NSKE changing in physical properties proved that the NSKE is physically stable. Igniting a sample of the NSKE proved it has a high flash point and hence could be mixed with kerosene and burnt in lanterns without addition of synergist nor humectant. Without these additives, the advantage of low mammalian toxicity was maintained as most of these additives are toxic. Besides burning in lanterns, the repellent can equally be sprayed or rubbed. Table 3 show that burning is a better method but spraying and rubbing could frequently be renewed after the active period. The repellent was found to be non-toxic to man and his domestic animals but highly toxic to the target mosquitoes.

Conclusion

In conclusion, the neem seed kernel extract is best for use in the production of mosquito (culicidae) repellent considering that it is cheap and readily available, non-toxic to man and his domestic animals and contains an appreciable quantity of oil. Moreover, the extract can be stored for a reasonable long time without going rancid. Repellent produced from neem seed kernel is active within a short time over a small range of area. The advantage of this is that it is confined to the user at the time he needs it. Moreover, being biodegradable, the repellent breaks away into harmless products within a reasonable time. By this, it cannot produce harmful effects on man nor pollute his environment (soil, water and air). The repellent is non-hazardous and non-toxic to man and his domestic animals but highly toxic to the target mosquitoes through the anti-feeding or phago-repellent action of the active component (Azadirachtin), a complex methyl ester, an unstable compound which transforms itself into four simpler methyl esters as confirmed by GC – MS analysis. These methyl esters are active in one way or the other, but 11, 14 – Eicosadienoic Acid has structure closest to azardirachtin. It is the most active because of the presence of two double bonds. Local production and use of this repellent could enhance the Nigerian content in phytochemical production, increase the Nigerian Gross Domestic Product hence advancing the Nigerian economy.

Recommendations

The following recommendations are made:

i. Considering the importance of neem in the control of insect pest which hampers economy, the government should set up an agency in the forestry depart-

ment to promote the plantation of neem trees in large numbers by setting up neem forests.

ii. Considering the deleterious effects of mosquitoes and other insect pests on the economy, the government should set up an industry for production of insect repellent from neem seed kernel extract in large capacity.

iii. To increase level of awareness on the advantages of the new product, government should organize an extension education service to educate the masses on the NSKE mosquito repellent.

iv. Besides the production of mosquito repellent, Neem Seed Kernel Extract (NSKE) can also be used for soap production, tooth paste, cream, waxes, lubricants and fuel; government should therefore encourage research on the use of NSKE for these products.

Nomenclature

Abbreviation	Definition
AC	Active Component
EPA	Environmental Protection Agency
FAO	Food and Agricultural Organisation
GC-MS	Gas Chromatography- Mass Spectrophotometry
IR	Infra-red
NMR	Nuclear Magnetic Resonance
NSKE	Neem Seed Kernel Extract
UNIDO	United Nations Industrial Development Organisation
UV	Ultra-Violet
WHO	World Health Organisation

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