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Evaluation of the repellent activity of *Azadirachta indica* oil (meliaceae) from Cameroon, and its preparations on *Anopheles gambiae, Culex quinquefasciatus and Aedes albopictus*

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Abstract

The emergence of synthetic insecticides resistant genes in mosquitoes has prompted researchers to develop vector control tools based on the use of natural repellent plants. Thus, in this study, the repellent activity of Azadirachta indica seed oil (Neem oil) as well as that of three of its preparations (candle, cream and lotion all at 30%), were evaluated on experimental populations and on wild populations of Anopheles gambiae s.l., Culex quinquefasciatus and Aedes albopictus. The repellent effect was assessed in the laboratory, and the percentage of protection duration were noted on the field. Under laboratory condition, Neem oil resulted in 48% repulsion against Culex quinquefasciatus, 10% against Anophelès gambiae and 6% vis-à-vis Aedes albopictus; with the lotion, there was 46% repulsion against Anopheleles gambiae, 25% against Culex quinquefasciatus and 10% against Aedes albopictus. The repellent effect of the cream, was more pronounced on Culex quinquefasciatus with 22% repulsion, versus 19% against Aedes albopictus and 3% against Anopheles gambiae. Candles showed 8% and 14% repulsion respectively against Culex quinquefasciatus and Anopheles gambiae. In the field, Culex quinquefasciatus was less sensitive to Neeem oil with a repellency rate of 89% and a protection time of 1hour and 30 minutes. He was even less sensitive to lotion with 30 minutes of protection for a repellency rate of 19%. No bite of Anopheles gambiae s.l. nor of Mansonia was recorded during the entire duration of the test by the volunteers who used Neem oil, that means a repellency rate of 100%. The oily solution (lotion) also recorded a 100% repellency rate against Mansonia sp and Anopheles gambiae for 2 hours 30mn and 3 hours respectively. Thus, Neem oil from Cameroon and its 30% lotion can be used as alternatives to synthetic insecticides in reducing mosquito-human contact and in reducing the density of vector populations.

Keywords: Repellent activity, Neem oil, *Anopheles gambiae s.l., Culex quinquefasciatus, Aedes albopictus*, vector controlI.

Introduction

Although there are a plethora of vector control methods, the majority of them are based on the use of synthetic insecticides, which is costly and harmful to the environment and to humans. Moreover, they are inaccessible to the majority of populations, and even those that are accessible are often used inappropriately.

In addition, the emergence of insecticide resistance genes in mosquitoes is a major problem facing malaria control programmes in most African countries.

It is therefore necessary to develop vector control tools using natural repellent plants in order to limit both the nuisance caused by biting insects and the transmission of vector-borne diseases. *Azadirachta indica* A. Juss (Meliaceae), commonly known as neem, is a plant that has long been in used in Indian traditional medicine for its insecticidal, fungicidal, larvicidal and antimalarial properties[1,2]. Called "Gagne" in the Far North and North regions of Cameroon, neem is widely used by people to treat skin and abdominal diseases, as fungicide and insecticide for the protection of growing vegetable plants. It is also said to have antibacterial and antiviral properties[3].

Some authors have also associated Neem oil with a "repulsive potential" against arthropods such as *Culex quinquefasciatus* and *Anopheles gambiae* s.l.[1,4]. Indeed, it can be used by direct application of "pure oil" or creams on the skin[5]. Similarly, in Cameroon some people use it as incense (by depositing neem oil on hot coals) in order to repel biting insects. The objective of this study was to evaluate the repellent activity of neem oil and some of its preparations against mosquitoes such as *Anopheles gambiae* s.l., *Culex quinquefasciatus* and *Aedes albopictus*.

Material and methods

Vegetable material: The seeds used (Figure-1) came from the fruits of the mongooses of Far North region of Cameroon, they were dried in mid-shade and cold pressed with an oil press.

Figure-1: Seeds of Azadirachta indica.

Material for the oil characterization: Balance (KERN, WIC1400338, MAX 4200g, sensitivity 0.01gr), pH meter (METLLER TOLEDO, serial number: B450351189).

Reagents; Distilled water, chloroform, NaOH 10%, concentrated H_2SO_4, potash solution 0.5N, ethanol 95, phenolphthalein 1%, acetic acid solution - chloroform (3:2), KI solution, sodium thiosulfate, starch poisoning, Alcoholic KOH solution 0.5N, Isobutanol-ethanol solution (v/v 1/1), HCl Solution 0.5N.

Materials for the manufacture of preparations: Candle wicks, candle molds, water bath, mortar, packaging pots, spatulas, beakers, breeding cages, test cages, mosquito net web, plastic translucent bins, kneading box, care gloves, erlenmeyers, elastics, vacuum cleaners, test cages, Mousti dose® 30% DEET (repellent spray, infested area 50ml), torches, containers for collection.

Extraction and characterization of oil: Seeds from Garoua (north-Cameroon) were sun-dried, then pressed and ground (cold pressure). The resulting crude oil was decanted in small aluminum futs and then bottled in plastic containers. The cakes obtained after pressing are also collected and weighed. The performance was calculated according to the formula

$$R = \frac{mb}{mi} X\, 100$$

With: *mb*: crude oil mass*: mi*: initial mass of seeds.

Physicochemical Characters of Oil: Organoleptic Properties: We observed the consistency, colour, flavour and smell of the oil.

Physical properties: Solubility: in a test tube, successively introduce 3ml of distilled water and 3 drops of oil and observe. In another test tube, put 3ml of chloroform then 3 drops of oil and observe. As oil and water are non-miscible, they separate in the tube, with water occupying the bottom and oil the top. On the other hand, because oil is soluble in chloroform and other organic solvents, it forms a homogeneous and clear mixture.

Density: Density is an important physical characteristic in the classification of oils. It is determined according to the AFNOR T60-214 standard. We used the weighing method. The weighing was done at 25°C. Weigh an empty insulin syringe and dry it. Fill the syringe with oil and weigh it. Note the mass (m). Do the same with water and note its mass (m_e). Density is determined according to the formula:

$$d = \frac{m}{m_e}$$

With: d: density of oil, m: weighed oil mass (g), m_e: mass of the same volume of water (g).

Chemical Properties: Characterization of Terpenoids. In a test tube, put 5ml of oil and then slowly add 3ml of concentrated H_2SO_4. The presence of terpenoids results in the formation of a red coloration [6].

Determination of different indices: Acid Index: This is the amount of potash, expressed in milligrams, needed to neutralize the free acidity contained in 1g of fat. Introduce into a 250ml erlenmeyer, 7g of oil and add 50 to 70ml of hot ethanol, previously neutralized by a few drops of KOH, then add 2 drops of phenolphthalein, title the contents of the bottle with a potash solution of known normality, stirring constantly until stable pink for 30 seconds. Note the volume Vp of potash used for the reaction mix; make 3 determinations on the sample and calculate the acid index using the following formula:

$$Ia = \frac{Vp \times N \times 56{,}1}{m} \quad (Ia \text{ in mg KOH/g oil})$$ [7]

With: N = normality of the ethanolic KOH solution used m = mass of the test intake.

A good quality oil must have zero or low acidity.

Peroxide index[7]: It measures the degree of fat rancidity after exposure to air. The latter causes the formation of peroxides from unsaturated fatty acids. Prepare a 250ml erlenmeyer containing 1g of oil and 20ml of the acetic acid-chloroform mixture 3:2 (test bottle); in another erlenmeyer introduce 20ml of the 3:2 acetic acid-chloroform mixture (control bottle); In each 1ml bottle of a solution obtained by dissolving 1g of KI in 1ml of distilled water; butcher, mix well and place it in the dark for 5 minutes; In each bottle, add 75ml of distilled water and stir; then title the iodine released by sodium thiosulfate in the presence of starch as an indicator. The peroxide index is determined according to the formula:

$$I_a = \frac{(V_E - V_T)\times 10}{m} \text{ (in mEq/g of oil)}$$ [7]

With: V_E = volume of sodium thiosulfate used for the test, V_T = volume of sodium thiosulfate used for the control, m = sample mass (g), Make three determinations and consider the average value.

Saponification Index: It is the number of milligrams of potassium hydroxide needed to saponify 1g of fat. This value allows us to estimate the lengths of the carbon chains of the fatty acids that make-up the oil. In a 250ml erlenmeyer, introduce 500 mg of Neem oil and 10ml of ethanol-isobutanol solution (v /v 1:1). In another erlenmeyer (control bottle), put 10 ml of ethanol-isobutanol (v /v 1:1); in each Erlenmeyer, add 20 ml of alcoholic potash of concentration 0.5mol/l; adapt the air chiller and put in the boiling water bath for 45 minutes, stirring; add 2 drops of phenolphthalein and dose excess potash with 0.5 mol/L concentration hydrochloric acid, stirring constantly until discoloration stable for thirty seconds. Note the volume of HCl (V_E) used for the test, and (V_T) the one used for the control[8].

$$I_s = \frac{(V_T - V_E)\times C\times 56{,}1}{m} \text{ (I_s in mg of KOH)}$$

With: V_E = HCl volume used for the test, V_T = HCl volume used for the control, C = concentration of the HCl solution, m = exact mass of the oil in gram.

Ester Index: It expresses in mg the amount of potassium hydroxide needed to saponify esters present in 1g of substance. This corresponds to the difference between saponification and acid indice. EI = IS – AI

Manufacturing of Galenic preparations: The concentration for our preparations was 30%, chosen on the basis of the effective minimum concentration of N N diethyl l3 Methylbenzamide (DEET), the most widely used repulsive in the world, in case of exposure to Anopheles mosquito[9].

Cream: According to the European pharmacopoeia, creams are multiphase preparations, made-up of a lipophilic phase and a watery phase. Hydrophilic creams whose external phase is the hydrophilic one should be distinguished from lipophilic creams whose external phase is the lipophilic one, as is the case for our cream[10].

Operating Mode, For the Neem Oil Cream: Weigh the ingredients separately for 200g of cream. In a mortar heated in a water bath, introduce the elements of the hydrophobic phase (oil and lanoline); maintain this phase at 30°C for 30 minutes. At the same time, prepare the hydrophilic phase by mixing water, glycerin and methyl paraben; bring this phase also to 30°C before incorporating it into the lipophilic phase under continuous agitation. Keep stirring until completely cooled and put in pots[11]. For the control cream that will serve as a negative control, proceed in the same way after replacing Neem oil with sweet almond oil.

Organoleptic control: It consisted of the appreciation of the smell, colour, and texture of the cream by applying a layer of the preparation on the back of the hand. The galenic control consisted of macroscopic examination with the naked eye.

Operating Mode, For the Neem Oil Cream: Weigh the ingredients separately for 200g of cream. In a mortar heated in a water bath, introduce the elements of the hydrophobic phase (oil and lanoline); maintain this phase at 30°C for 30 minutes. At the same time, prepare the hydrophilic phase by mixing water, glycerin and methyl paraben; bring this phase also to 30°C before incorporating it into the lipophilic phase under continuous agitation. Keep stirring until completely cooled and put in pots[11]. For the control cream that will serve as a negative control, proceed in the same way after replacing Neem oil with sweet almond oil.

Table-1: Formula of Neem Oil Cream.

Neem oil cream			Negative control cream	
Neem oil : 30%	Active Ingredient		Almond oil 30%	Oily base
lanoline: 23%	Sel emulsifying		lanoline: 23%	Sel emulsifying
glycerin: 3%	Moistering		glycerin: 3%	moistering
methyl paraben: 0,4%	Preservative		methyl paraben: 0,4%	Preservative
Water eqf 100	moistering		Water eqf 100	moistening

eqf : sufficient quantiyt for…

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pH measurement. Calibrate the pH-meter by dipping the electrode successively in three buffer solutions pH: 7.0, 4.0 and 10.0 solutions respectively. After calibration, rinse the electrode in distilled water. In a beaker, add 50g of cream and 10ml of distilled water; homogenize, measure the temperature and the pH.

Lotion: According to the European pharmacopoeia, liquid preparations for skin application are variable viscosity preparations used for local or transdermal release of active substances. The lotions are intended to be applied to the injured or uninjured skin. These are emulsions or suspensions that contain one or more active ingredients in an appropriate vehicle [12]. For the 30% oil lotion, we mixed neem oil and sweet almond oil in 3-7 proportions at 30° C for 10 minutes.

Candles: A candle is composed of two main elements: the body of the candle (wax) and a braided wick (usually cotton)[13]. The first step in making candles is the pre-waxing of the wicks: for 100 grams of candle, melt 15g of wax in a water bath at 70°C; at the same time, bring 4.5g of neem oil to the water bath at 30°C for 10 minutes; mix the two liquids and dip the strands in them; allow to cool and insulate the strands.

For the second step (making candles), install a pre-waxed wick in the middle of each mold and hold it with a needle perpendicular to the mold. Weigh 30g of Neem oil and 70g of wax and put them in a water bath at 70°C for 10 minutes. Mix the two and quickly pour into the molds, then let cool, unmold and check the quality of the product. This control involves the visual control of the candle as well as the analysis of the flame's behaviour during combustion, the shape of the flame, the shape of the wick, the extinction of the wick after the candle is blown; the tests were repeated 4 times during different combustion cycles. For control candles, follow the same protocol, using wax alone.

Obtaining adult females mosquitoes: The larvae of Aedes sp, Anopheles sp and Culex sp were collected at several sites in the city of Yaounde. The larvae were raised in bins and fed on semolina from biscuits and shrimp. The nymphs were sorted every day. Larvae and nymphs were exposed to the sun during the day, and shaded in the evening to respect their nycthemeral rhythm. The nymphs were re-sorted before the bins were stored, and the nymph pots were placed uncovered in cages for emergence. This operation was repeated every day until the environment was exhausted into nymphs and their water was changed after 2 or 3 days. Adults were fed with a 10% glucose solution, soaked on cotton. The laboratory-obtained Aedes eggs were soaked in spring water and exposed to the sun. After hatching, the larvae were transferred to bins.

Evaluation of the Repulsive Activity: Entomological Test in the Laboratory. The Bigoga model was used for the evaluation of repulsive activity[14]. The device used included a 324cm 2 cage (A) connected to a cage (B) of the same size by a 10 cm white joint (Figure-2). Neem oil, cream and lotion were used for the coating of cage A while DEET, sweet almond and white cream were used for the coating of the treated cage. The coatings were then left at the ambient air for 30 minutes and then, 25 female mosquitoes aged 5-7 days were inserted into Cage A using a vacuum cleaner. Mosquito behaviour was observed and the number of mosquitoes passing from cage (A) to cage (B) was recorded every 30 minutes for 4 hours. Each test was reproduced 4 times for the formulations (oil, cream, lotion and Neem candle), 2 times for positive control (DEET), 2 times for sweet almond oil and 2 times for cream with sweet almond oil.

Figure-2: Laboratory Repulsive Activity Assessment Device.

For the candles, the procedure was the same: the lit candle was in the cage (A). For each species of mosquito the test was repeated twice for Neem candles, and 2 times for the negative control of the candles.

Field testing: human landing catch method: The method used is based on the WHO protocol for the effectiveness of repellents for skin use[15]. Before the tests began, an ethical clearance was issued to us by the Institutional Ethics Committee of the "University des Montagnes" (N°2017/168), informed consent forms were completed and signed by each volunteers and anti-malarial prophylaxis was administered to them following the recommendation of the national malaria control program.

Human landing catch method took place outside a house in the Mvan district of the Yaoundé city. We tested only the preparations that were selected in the laboratory as having interesting properties: Neem oil and its 30% lotion. Volunteers were selected and trained to catch mosquitoes before they bit (Figure-3).

We used 5ml aliquotes uniformly coated on both legs of the volunteer. One of the volunteers was treated with Neem oil, the second with 30% lotion, the third (positive witness) treated with DEET, and the fourth (negative volunteer) with sweet almond oil and the fifth received no treatment. They were positioned at sites far away from the house. The collection period was 3 hours and observations were made at 30 minute intervals between 8 p.m. and 11 p.m.

All mosquitoes that landed on the exposed low limbs of each volunteer during each considered time interval, were captured and transferred to the storage container for subsequent counting and species identification. To each of the volunteers, was assigned a container corresponding to a 30-minute interval. One test included the random rotation of each test volunteer, the positive control and the negative control at all collection sites at one-hour intervals. The test was reproduced twice. The percentage of repulsion R_P on the field trials was determined for each hour of the test as follows:

Figure-3: human landing catch methodaptureur lors du test sur appâts humain.

$$R_p = \frac{C-T}{C} \times 100$$

Table-2: Extraction yield and physic-chemical traits of Neem oil.

Quantity of dried seeds	9,23 kg
Weigh of oil after decantation	2,50kg
Weigh of alleau	6,69 kg
Imput	27%
organoleptic tests	Strong characteristic odour, bitter, liquid at ambient temperature, light brown
Emulsions and solubility	Formation of an emulsion with NaOH, Insoluble in water, chloroform soluble
Density	0,927 at 25°C
Terpenoïd	Present
Acid index ($mg.g^{-1}$)	2,60
Saponification Index ($mg.g^{-1}$)	199 ,71

The products obtained: The products obtained and tested are shown in Figure-4. The resulting cream was yellowish, neutral in smell, smooth in appearance, creamy to the touch, with a good spread on the skin. It was homogeneous and stable with a pH of 8.41. The lotion was a homogeneous and stable mixture that spreads well over the skin leaving an oily residue. The candles were smooth, golden in colour. Observation of the flame's combustion behaviour revealed a conical flame of about 3cm, a slightly curved wick, an easy extinguishment of the wick after blowing and an odour of "oil on fire" during combustion.

Mosquito breeding: Mosquito larvae were collected at several sites in the city of Yaounde including Mvan, Tropicana Emombô 2, Nkomo and Essomba. Once the larvae were collected, the nymphs were formed over the days due to the diversity of larval stages in the same deposit (eggs, stage 1, 2, 3, 4 and nymph larvae). Every day, we sorted nymphs and exposed larvae to the sun until the nymphal stage reached. The adults were obtained about 2 days later. The eggs collected in the field hatched 2 days after immersion in the water and the larvae were transferred to bins. By the end of their development, they had turned into nymphs and 2 days later, we were getting adults that we left 5 days to mature while feeding them with sweet water before moving on to the experience of the test in cage.

Evaluation of repulsive activity: Entomological tests in the laboratory: A total of 450 mosquitoes were used to perform tests for each species, 1350 mosquitoes in total, at a rate of 25 mosquitoes per test, each test being repeated 4 times for Neem oil, cream and lotion; 2 times for positive control (DEET), sweet almond oil and for cream with almond oil.

Figure-4: Various products tested: Neem oil (a), lotion with 30% Neem (b), cream with 30% Neem (c), candle 30% Neem (d), positive DEET (e), and negative controls (sweet almond oil (f), cream with sweet almond oil (g) and beeswax candle (h).

Anopheles gambiae s.l.: Of the products tested on *Anopheles gambiae*, the 30% lotion recorded the highest rate of repulsion with maximum effect from 3hours 30min (46%). Neem oil and cream had a repulsion rate of 10% and 19% respectively, with maximum activity observed after 1hour 30min and 3hours30min exposure. DEET, control cream and sweet almond oil recorded lower repulsion rates of 6, 2 and 2% each after 1 hour and 30 hours of exposure (Figure-5). There was a significant difference between each of the Neem-based preparations and its negative (wilcoxon test: Neem oil/almond oil: p = 0.012; Lotion 30% / almond oil: p = 0.012; Cream 30% / Control Cream: p = 0, 011).

Neem Oil, Lotion and DEET induced increasing mortality of *Anopheles gambiae* s.l. specimens remaining in the treated cage (Figure-6). The maximum mortality rate was reached between 3h30 and 4 hours of exposure: 55% for Neem oil, 27% for lotion and 94% for DEET. The evolution of mortality induced by Neem oil and DEET was statistically different (χ^2 = 4,79; p = 0, 028).

Culex quinquefasciatus*:* For *Culex quinquefasciatus*, Neem oil achieved the highest rate of repulsion with maximum effect at 2h30min (48%). Cream and lotion at 30% had a repulsion rate of 22 and 25% respectively, with maximum activity observed after 2h30 min and 3h30 min exposure. In contrast, a low rate of repulsion (6%) was observed for DEET and almond oil after 2 hours of exposure, as well as zero repulsion for the control cream (Figure-7). There was a significant difference between each of the Neem preparations and its negative control (Wilcoxon test: Neem oil / almond oil: p - 0.011; Lotion 30% / almond oil: p - 0.010; Cream 30% / Control cream: p - 0, 011).

As with *Anopheles gambiae* s.l, losses were recorded among the specimens of *Culex quinquefasciatus* remained in the treated cage at lower rates (Figure-8). Maximum mortality rate after exposure to Neem oil (11%) was reached after 2hrs30 min a.m. The DEET reached a rate of 94%, 2 hours after the start of the test. However, there was no significant difference between the mortality induced by these two products on Culex (χ^2 = 3, 58; p = 0,06).

	Control cream	Almond oil	DEET	Cream 30%	Lotion 30%	Neem oil
0	0	0	0	0	0	0
30 Min	0	4	0	8	0	0
1 H	2	3	3	10.3	29	9
1 H 30	2	2	6	16	38	10
2 H	2	3	6	18	39.5	10
2 H 30	2	3	6	19	42	10
3 H	2	3	6	19.5	43.5	10
3 H 30	2	3	6	19	46	10
4 H	2	3	6	19.5	46	10

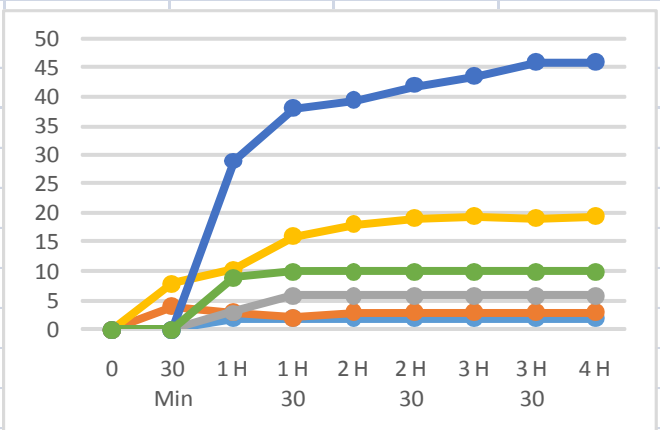

Figure-5: *Anopheles gambiae s.l.* repulsion rate based on exposure time.

exposure time	Neem oil	Lotion	DEET
0	0	0	0
30 Min	12	3	21
1 H	18	13	50
1 H 30	23	18	61
2 H	30	20.3	68
2 H 30	39.1	21.5	81
3 H	42	22	90
3 H 30	55	27	94
4 H	55	27	94

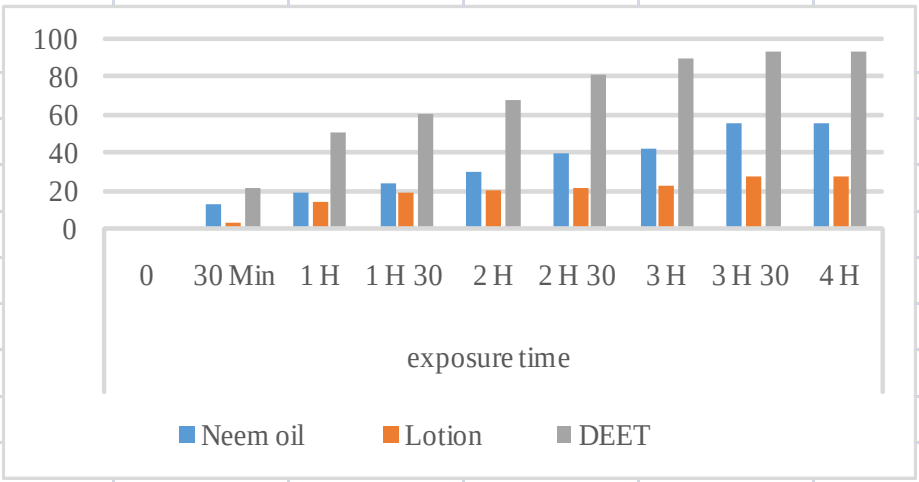

Figure-6: *Anopheles gambiae* mortality based on exposure time.

Aedes Albopictus: Of all types of mosquitoes tested, no specimen returned to the treated cage after leaving it, other than those of *Aedes Albopictus*, which therefore recorded the lowest sensitivity to the products tested in terms of repulsion (Figure-9). Thus, rates of 3%, 6% and 10% were recorded respectively with cream, oil and lotion after 1 hour, 3h30 and 3 hours of exposure. Similarly, very low repulsion rates were recorded with DEET, control cream and sweet almond oil (2%) 3hours of exposure. Nevertheless, there was a significant difference between each of the preparations and its negative control. (Wilcoxon test: Neem oil / sweet almond oil: p = 0.017; Lotion 30% / sweet almond oil: p = 0.011; Cream 30% / Control cream: p = 0, 033).

exposure time	Control cream	Almond oil	DEET	Cream 30%	Lotion 30%	Neem oil
0	0	0	0	0	0	0
30 Min	0	7	5	9	16	43
1 H	0	8	6	13	21	45
1 H 30	0	7	5	19	21	45
2 H	0	6	6	20	23	47
2 H 30	0	6	6	22	23	48
3 H	0	6	6	22	23	48
3 H 30	0	6	6	22	25	48
4 H	0	6	6	22	25	48

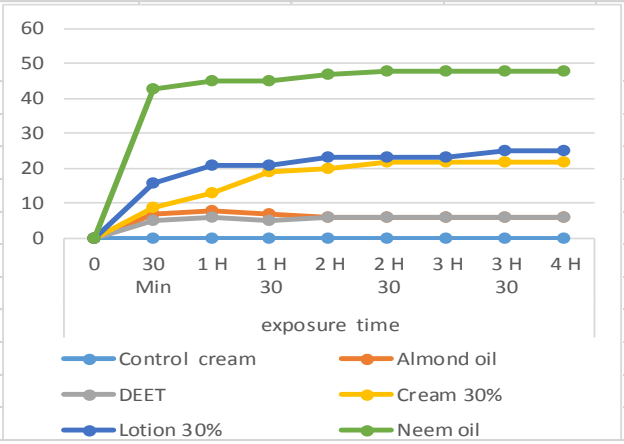

Figure-7: Culex quinquefasciatus repulsion rate based on exposure time.

exposure time	DEET	Neem oil
0	0	0
30 Min	5	0
1 H	19	0
1 H 30	63,5	7
2 H	94	7,8
2 H 30	94	11
3 H	94	11
3 H 30	94	11
4 H	94	11

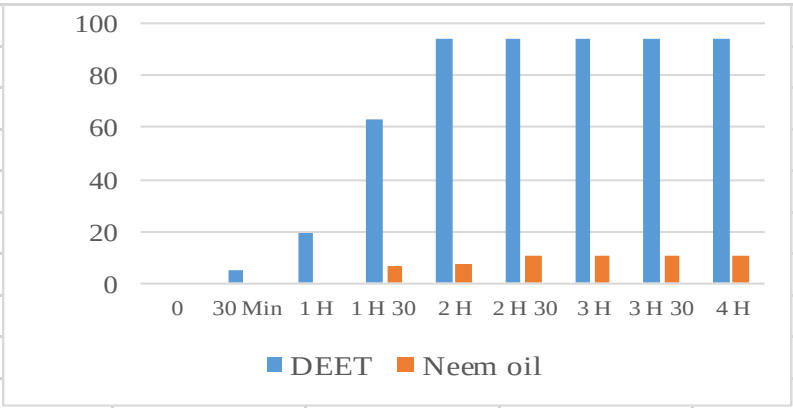

Figure-8: Mortality rate of *Culex quinquefasciatus* according to exposition time.

exposure time	Control cream	Almond oil	DEET	Cream 30%	Lotion 30%	Neem oil
0	0	0	0	0	0	0
30 Min	0	0	0	0	4,5	4
1 H	0	1	0	3	5	5
1 H 30	0	1	0	2	7	5,5
2 H	0	2	2	2	7	5,5
2 H 30	0	2	2	2	8	5,5
3 H	0	2	2	1	10	5,5
3 H 30	0	2	2	1	9	6
4 H	0	2	2	2	9	6

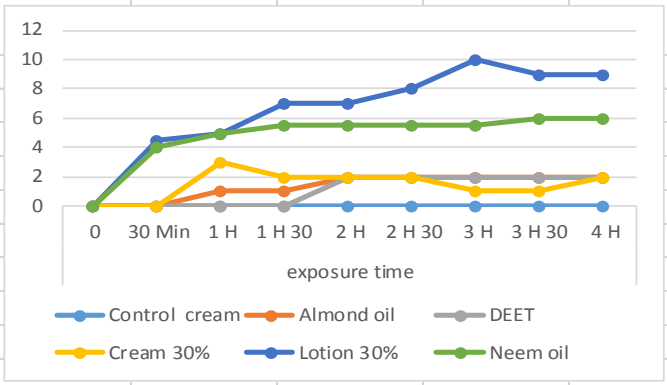

Figure-9: *Aedes albopictus* repulsion rate based on exposure time.

In the tests with *Aedes Albopictus*, a higher mortality was recorded, compared to that obtained with *Culex quinquefasciatus*. The maximum rate of 25% was reached at 3hours for Neem oil. DEET reached similar rates at 1 hour with a maximum rate of 98% reached at 3hours 30min (Figure-10). In addition, there was a significant difference between neem oil-induced mortality and DEET ($X^2 = 7.01$; $p = 0.008$).

Candles: A total of 300 mosquitoes were used to perform the tests for each species, making 900 mosquitoes in total, at a rate of 25 mosquitoes per test, each test being repeated 2 times for the Neem candle, and 2 times for negative control (candle made only of wax). During the burning of the Neem candle, 14% of specimens of *Anopheles gambiae* s.l. and 8% of *Culex quinquefasciatus* specimens escaped from the treated cage. No changes were noted with *Aedes albopictus*. No movement was observed during the burning of the control candle.

Field test: human landing catch method on volunteers: During the two nights of field collection, 130 mosquitoes were caught in total (Figure-11). The specimens consisted mainly of: 101 *Culex quinquefasciatus* (77%), 15 *Mansonia sp* (12%) and 14 *Anopheles gambiae* s.l. (11%). However 40 mosquitoes were collected by the control treated with sweet almond oil and 34 by the negative control. In addition, 52 mosquitoes were collected by the volunteer treated with lotion and none was collected by volunteers treated with Neem oil and DEET (on the first day) against 4 by the volunteers treated with Neem oil and none by the ones treated with DEET (the second day).

exposure time	Neem oil	DEET
0	0	0
30 Min	0	3
1 H	0	28
1 H 30	9	40
2 H	12	78
2 H 30	16	91
3 H	25	93
3 H 30	32	98
4 H	34	98

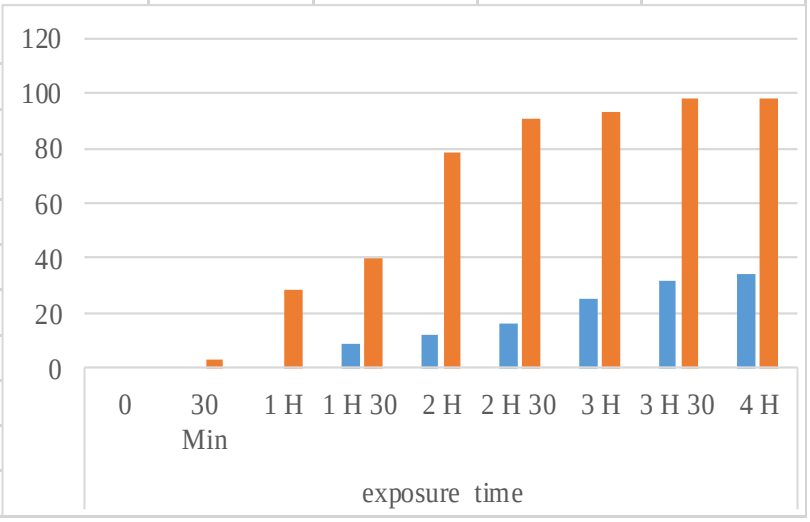

Figure 10***:*** Mortality of *Aedes albopictus* according to exposition time.

	Neem oil	Lotion 30%	DEET
Culex	89	89	100
Anopheles	100	100	100
Mansonia	100	100	100

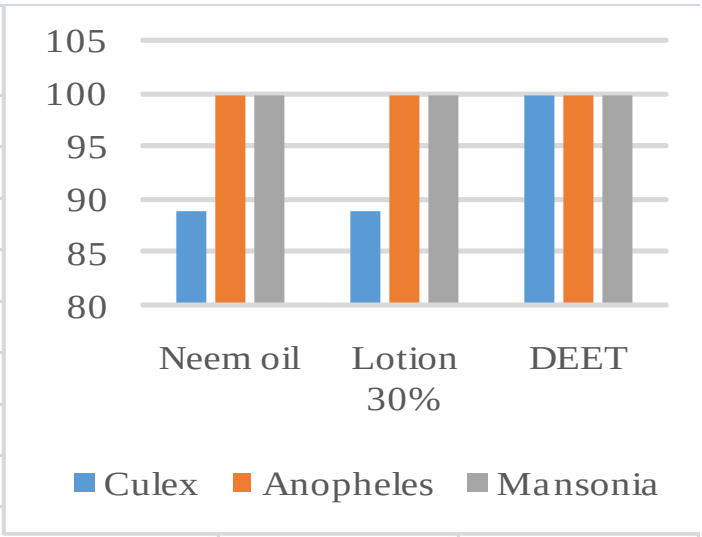

Figure-11: Mosquito specimens collected from negative controls and treated during both tests on the field.

Protection provided by the various preparations: The rate of protection provided by Neem oil and lotion is higher with A*nopheles gambiae* s.l. with 100% protection for the 3 hours duration of the test. A 100% protection rate was also recorded with Mansonia sp, after application of Neem lotion and DEET, providing complete protection for 2 hours and 3 hours respectively. The effect on *Culex quinquefasciatus* was the least marked with a protection rate of 89% and the lowest protection time recorded among test products, less than 30 minutes.

Discussion: Neem oil was obtained by cold pressure with an extraction yield of 27%. This result is close to that of Sagoua who obtained an extraction yield of 28%[16]. The cold mechanical pressure extraction process does not achieve a very high extraction yield, but avoids alterations of the oil unlike the extraction by Soxhlet, for which a higher yield would probably have been obtained but using chemical products and heat that could alter the quality of the oil. The resulting oil had a light brown hue, similar to the orange-brown hue obtained by Sagoua but which differs from that of Neem oil from Senegal (light green colour) and that of dark green Neem of India oil[16]. In addition, the sulphuric acid test revealed the presence of terpenoids, corroborating the results of Adjanohoun and Bashige[6,17].

During the cage tests, after 4 hours of exposure to the various products, the specimens escaped from the treated cage did not return back to it, except for *Aedes Albopictus*. The repellent effect was gradually expressed throughout the tests with *Culex quinqefasciatus* and *Anopheles gambiae* with 48% repulsion rates obtained with oil, 2h 30 min after the start of the tests, and 46% obtained with lotion 4 hours after the start of the tests. Throughout the experiment, the specimens of *Culex quinqefasciatus* and *Anopheles gambiae* that passed into the untreated cage were relatively calm, unlike those that remained in the treated cage, rather agitated and dizzy, especially *Anopheles gambiae* s.l. This could be due to contact with the tested products.

The cream showed only low rates of repulsion, although the differences between the control and the cream were significant. This could mean that the cream would be active, but that it had less effect as a result of modification or imprisonment of the active ingredient by other ingredients. This seems all the more true since a study with another Neem oil-based cream yielded interesting results: 78%, 89% and 94.4% protection against the genus Aedes, Culex and Anopheles respectively[5].

Mortality rates of 55%, 25% and 11% were recorded with Neem oil respectively on *Anopheles gambiae, Aedes albopictus* and *Culex quinquefasciatus*, as well as a 27% mortality rate, recorded with lotion. Lower adult mortality rates were announced by Agbizounou testing the efficacy of vegetable oil extracts on *Anopheles gambiae s.l.* Giles and *Culex quinquefasciatus* Say resistant to pyrethroids. The high larvicide effect he observed with *Azadirachta indica* oil suggests that it could be used to control resistant mosquitoes including *Anopheles gambiae*, the main vector of malaria in Benin [18]. The oil and lotion had their characteristic smell preserved unlike the cream; this tends to confirm Tahiri's statement that contact and inhalation are the two essential pathways to the effectiveness of Neem[19]. It should be noted, however, that Agbizounou observed a low toxicity by contact (mortality rate=35%) and simultaneous action by contact and ingestion (mortality rate approaching 100% from the first days) concluded that the pathway by ingestion would be the most toxic but a synergy of contact-ingestion was suggested[18]. Neem candles had a lesser effect on the specimens tested, which is inconsistent with data collected from Indian traditional medicine that using 1% Neem oil in an oil lamp would reduce the incidence of Malaria, moving the Anopheles away[20]. During field tests, the effectiveness of Neem oil and 30% lotion is more important vis-à-vis Anopheles gambiae s.l.: 100% protection for the 3 hours duration of the test. This is consistent with the results of Abiy, which announced a protection time of 3 hours with a 20% Neem lotion [4]. We have recorded a 100% protection rate with Neem lotion and oil vis-à-vis Mansonia sp collected from the field, with complete protection for 2 hours 30min and 3 hours respectively. A less pronounced effect on *Culex quinquefasciatus* was observed, with a protection rate of 89% close to the results obtained by Mandal, who recorded a protection rate of 90.26% with Neem oil vis-à-vis this arthropod. Unlike our oil which allowed less than 30 minutes of protection, theirs gave complete protection for 3 hours[1]. This could be due to the individual susceptibility of each species and subspecies of mosquito, as well as the concentration of active compounds of the product tested[4]. This difference in effect on Anopheles sp and Culex quinquefasciatus was also observed by Dua, who noted a repulsion rate of 94.4% and 89% respectively when evaluating the repellent action of a Neem-based product against mosquitoes [5].

Conclusion

This study aimed to evaluate the repellent activity of neem oil and three preparations based on neem oil (lotion, cream and candle) on mosquitoes of the genus *Anopheles gambiae s.l.*, *Culex quinquefasciatus* and *Aedes albopictus*. After extracting the oil and formulating the preparations, we submitted them for experimentation on laboratory specimens and wild ones.

In laboratory conditions, the products tested demonstrated a repellent effect, to varying degrees depending on the species: 48% of Culex quinquefasciatus specimens, 46% of Anopheles gambiae s.l. specimens and 22% of *Culex quinquefasciatus* specimens escaped from the cage treated respectively with Neem oil, lotion and cream. On the other hand, candles caused 8% and 14% repulsion to specimens of *Culex quinquefasciatus and Anopheles gambiae.*

In addition, contrary to our forecasts, insecticide activity was recorded with mortality rates of 55%, 25% and 11% observed

during tests with Neem oil respectively on *Anopheles gambiae* s.l., *Aedes albopictus* and *Culex quinquefasciatus*; as well as a 27% rate observed with lotion only on *Anopheles gambiae* s.l. DEET, on the other hand, achieved a mortality rate of 98% (with *Aedes Albopictus*).

On the field, *Culex quinquefasciatus* was less sensitive to Neem oil with bites recorded in volunteers after 1hr 30min, let alone lotion that protected the latter's stings for only 30 minutes. Mosquitoes of the genus *Anopheles gambiae and Mansonia* were unable to bite or land on the legs of volunteers who had been coated with Neem oil for the duration of the 3 hours. Volunteers who used the lotion were also protected from Mansonia sp stings for 2hrs 30min, as well as from those of *Anopheles gambiae* s.l. for the duration of the test. Similarly, DEET provided 100% protection throughout the testing on field specimens.

Although the products tested had less repellent and insecticide effects than synthetic repellent (DEET), the results obtained open-up a field of possibilities for the formulation of effective repellents against mosquito bites from locally available plants such as *Azadirachta indica* A. Juss.

Neem oil from Cameroon and its 30% lotion can be used as alternatives to synthetic insecticides, for the reduction of both the mosquito-to-human contact and vector population density. However, further work is needed to refine the results. We think of testing neem candles made using paraffin instead of wax. We also think of the exploration of the insecticide activity of neem oil and the improvement of formulations.

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