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Full Length Research Paper

Toxic effects of neem products (*Azadirachta indica* A. Juss) on *Aedes aegypti* Linnaeus 1762 larvae

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Treatment and comparative analysis of the properties of aqueous extracts of seed kernel of *Azadirachta indica* A. Juss (neem) was carried out on *Aedes aegypti* larvae. The aim of this work was to evaluate lethal effects of neem products (1% Suneem, formulated neem oil and neem powder) on *A. aegypti* larvae. Assays showed that *A. indica* was toxic to larvae of *A. aegypti*. For 1% Suneem, 1% formulated neem oil and neem powder, the lethal concentrations and lethal time at 50% (LC_{50} and LT_{50}) for *A. aegypti* were 2 and 8 mg/l after 24 h and 3 mg/l after 120 h, respectively. Assays showed that Suneem and Formulated neem oil were more toxic to *A. aegypti* than Neem powder. Both products of the neem (*A. indica*, A. juss) have a remarkable influence on the development of *A. aegypti* larvae, causing an inhibition of nymphs and adults emergency. The Histopathological results revealed a serious damage on the epithelial columnar cells, a perturbation of alimentary flow, slightly hypertrophied cells, a beginning of vacuolisation on apical level, and a bursting of some cells in posterior part of the gut. However, nuclei, adipose tissue and muscles seem to keep normal appearance.

Key words: *Azadirachta indica* (neem), *Aedes aegypti* (mosquito), LC_{50}, emergency, histopathology.

INTRODUCTION

Mosquitoes in general and *Aedes aegypti* in particular constitute a major problem of public health and lead to serious human diseases such as malaria, encephalitis, yellow fever, dengue, hemorrhagic fever, filariasis and arbovirosis. Kettle (1995) quoted by El hag et al. (1999) reported that the instars larvae of mosquitoes have traditionally been affected by an application of synthetic chemical insecticides in solution of oil as emulsion or wettable powder. Nevertheless, repeated use of insecticides leads constantly to the risk of contamination of water used for domestic purposes, animals and humans by pesticides residues. It is also important to note the high cost of chemical pesticides and the development of resistances phenomena of much mosquito species vectors (Sivagnaname and Kalyanasundaram, 2004; Konan et al., 2003; El Hag et al., 1999).

The interest in developing biopesticides with natural origins such as azadirachtin has increased during the recent years because of the drawbacks of synthetic chemical pesticides (Tianyun and Mulla, 1999). *Azadirachta indica* (neem) belonging to the family of meliaceae, had insecticidal activity (Aliero, 2003). The neem

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Abbreviations: LC, Lethal concentration; Sol, Solvesso; LT, lethal time; F.N.O., formulated neem oil; LT, lethal time ; N.P., neem powder.

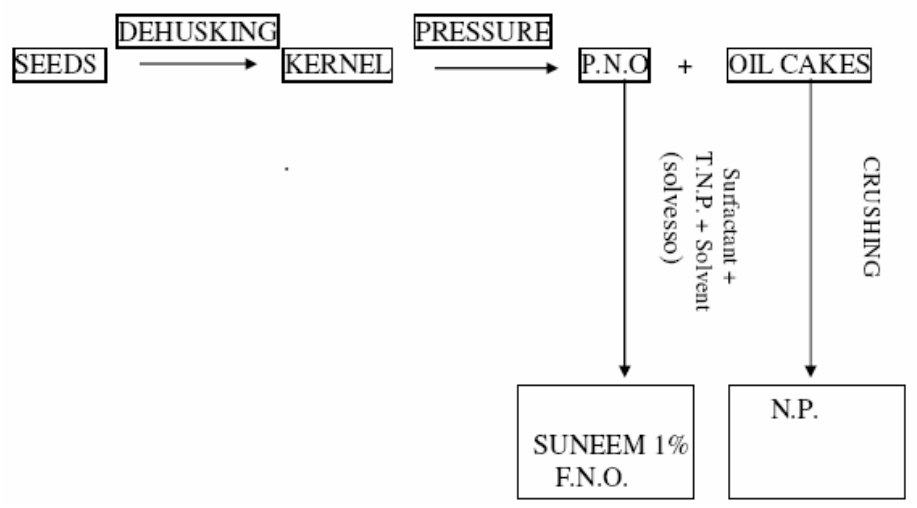

Figure 1. Process to obtain the neem products (*Azadirachta indica*, A. Juss*)* (Provided by the SENCHIM chemistry's industry Dakar- SENEGAL). P.N.O., pure neem oil; F.N.O., formulated neem oil; T.N.P., technical neem powder; and N.P., neem powder.

contains several biologically active constituents such as azadirachtin (Naganishi, 1975). The azadirachtin and others related compounds in neem products exhibit various modes of action against insects such as toxics effects (Azmi et al., 1998; El hag et al., 1999; Scott and Kaushick, 2000; Aliero, 2003; Wandscheer et al., 2004; Scott and Kaushick, 2000), antimitotic effects (Salehzadeh et al., 2003), antifeedancy, growth regulation, fecundity suppression and sterilization. In fact, azadirachtin has harmful effects on endocrinien system of an insect of Coleoptera *Epilachnus varivestus* and caused sterility in *Epilachnus varivestus* ects females (Schumuthere et al., 1981). Aliero (2003) reported that azadirachtin deteriorated the cuticle of larvae preventing them from moulting.

Based on the encouraging research on azadirachtin and others related compounds for control agricultural pests, it is desirable to investigate the potential usefulness of neem products on mosquito control. In the effort to explore biological effects of neem products, the current research was initiated to investigate the toxicity, histopathologicals and growth retarding effects on last instars larvae of *A. aegypti*.

MATERIALS AND METHOD

Experimental insect

A. aegypti Linnaeus 1762 (*Diptera*: *Culicidae*) was taken as experimental insect because of its role as a vector of pathogens of Yellow and Dengue fevers. Larvae of *A. aegypti* were collected in a plastic bottle 10 x 10 x 7 cm from the vat of culture of mosquitoes (artificials habitats) of the Department of Animal Biology in the Faculty of Sciences in University Cheikh Anta Diop of Dakar. The mosquitoes larvae are fed with bread powder under the laboratory conditions, following a temperature of 24°C +/- 1°C, a relative humidity of 80% +/- 3% and 11/13 h (light / dark) photoperiod.

Neem products

The test materials were two experimental formulations of neem products (1% Suneem and 1% formulated neem oil), a wettable powder (0.3% neem powder). This neem products as well as their manufacturing process have been provided to us by SENCHIM, an industry of Senegal (Dakar, Senegal) (Figure 1).

Toxic and growth retarding effects tests

The toxics effects are measured using five differents concentrations of each compound selected. These concentrations have varied from 2 - 10 mg/l for Formulated Neem Oil and Suneem and from 3 - 15 mg/l for Neem Powder. Twenty-five (25) late fourth instars larvae of *A. aegypti* are introduced in each square plastic bottles 10 x 10 x 7 cm and exposed to various concentrations of neem for either 23 - 25°C, with 11 / 13 h (dark / light, photoperiod) and 60 - 80% (Relative Humidity), in 500 ml of distilled water.

However, twenty-five (25) larvae of *A. aegypti* are exposed in 500 ml of distilled water with 0.01 ml of Solvesso (solvent used in formulation of neem products) (solvent control) (i) for treatment with 1% Suneem and 1% formulated neem oil (F.N.O.) or only 500 ml of distilled water in plastic bottle (blank control) (ii) for treatment with neem powder (N.P.). During the experimentation, both larvae are fed with bread powder. The experimentation with the larvae are replicated three times.

Larvae affected by the effect of neem, descended to the bottom of the bottle. Considered like dead are all larvae that do not move or do not answer to an excitation and leaving the ones that can make clear movements. Observations of the mortalities of the larvae are made daily and results noted.

In order to determine the effect of neem in the delay of the larvae growth, the same treatment is applied and the number of nymphs and adults emergence is registered every 24 h.

Data analysis

The results of the bioassay experiments conducted in the laboratory were analyzed as percent mortality and corrected for control mortality with Abbott's formula (Hubert, 1992):

Table 1. Percentage mortality of *A. aegypti* treated with 1% Suneem.

1% Suneem concentrations (mg/l)	Time of treatment (h)				
	24 h	48 h	72 h	96 h	120 h
Control	0	4	6	6	6
2	50	70.94	74	73.99	73.99
4	57	61.66	73.45	77.49	78.51
6	94.5	94.43	94.43	94.43	94.43
8	96.5	96.47	96.47	96.47	96.47
10	100	100	100	100	100
LC 50	2 mg/l (24 h)				

% M = [(N.D.L.T. – N.D.L.C.)/(Total N.L. - N.D.L.C.)] x 100

Where % M = percent mortality; N.D.L.T. = number of dead larvae during treatment of neem products; N.D.L.C. = number of dead larvae in the control (untreated); Total N.L. = total number of larvae used in each treatment.

The effect of neem products on *A. aegypti* larvae was analyzed by determining the significant level of mortalities results with ANOVA paired t-test and by comparing toxicity of neem products used in current studies with “Khi-deux” test.

Histopathological effects

The moribund larvae during the treatment and untreated larvae are taken and fixed in Carnoy 2 during 72 h. The dehydratation of tissue is made with two baths of 95% alcohol (ethanol) for 4 or 5 h. The larvae of *A. aegypti* are placed in two baths of butanol for 4 h. Then the larvae are impregnated by a bath of butyparaffin and included in the pure paraffin. Some blocks are achieved with the bars of Leuckart, and then are built with a scalpel. Some cuts are done with the "Minot" microtom models "Stiasnie". The cuts are glued on a blade. The coloration is made according to the technic of Trichome of Masson Goldner variant described by Martoja and Martoja (1969). At the end of the coloration, the cuts are recovered with a balm of Canada. The blades are dried at the steam-room (60°C). The observation of the blades is made by the photonic microscope "Motic" connected to a computer, permitting us to get photographs of the midgut cells of the treated and untreated larvae of *A. aegypti*. Histopathological effects are been studied in anterior, middle and posterior parts of the gut of *A. aegypti* larvae.

RESULTS

Toxic effects of neem products on *A. aegypti*

The toxic effects of the neem products on mosquito larvae are presented in Tables 1, 2, and 3. The neem products (1% formulated neem oil, 1% Suneem, and 0.3 % neem powder) extracted from the seed kernel of *A. indica* have shown larvicidal activity on *A. aegypti* mosquitoes.

A. aegypti is a hematophagous mosquito and vector of diseases like dengue and yellow fever. We have measured the effect of the neem on four instars larvae of *A. aegypti* by determining the mortality rates to 50 and 100%. After we have exposed larvae of *A. aegypti* to various concentrations of the neem products (Suneem, formulated neem oil and neem powder) for 120 h, the mortality of four instars larvae of *A. aegypti* increased significantly according to the concentrations (ddl = 4; F = -6.708; P = 0.0026 < 5 %) and the time of exposure to Suneem (ddl = 4; F= -1.623; P = 0.017 < 5%) (Tables 1, 2, and 3).

After five days of treatment, the average of mortality of *A. aegypti* larvae is 74.85%. In the control, the highest mortality is reached at 6%. Mortalities of the larvae in all of the concentrations used are very significantly different compared with those obtained in the control (ddl = 4; F = 81, 97; P < 0.001). The fourth instars larvae of *A. aegypti* are very sensitive to the various concentrations used in 1% Suneem. Suneem was found to be effective against early and late fourth instar larvae of *A. aegypti*.

The toxic effects of Formulated neem oil tested on *A. aegypti* larvae are shown in Table 2. The formulated neem oil killed *A. aegypti* larvae to 50% at the concentration of 8 mg/l (CL_{50}). This larval mortality at 50% occured after a very short time 24 h (TL_{50}). Moreover, *A.aegypti* larvae had 100% (LC_{100}) mortality in all of the concentrations in this current study at 144 h (TL_{100}). The mortalities of fourth instars larvae of *A. aegypti* varied significantly with time (ddl = 4; F = -4.305; P = 0.0126 < 5%). This significant variation of the mortality of *A. aegypti* larvae is in increasing direction following the different concentrations used (ddl = 4; F = -7.522; P = 0.00017 < 5%). The percent mortality produced in all of the concentrations from 24 - 144 h was significantly different from that of the control (6%) (ddl = 5; F = -11.58; P = 0.0015 < 5%). Treatment of *A. aegypti* larvae by The formulated neem oil was found to be very effective against fourth instars larvae of *A. aegypti* than 1% Suneem.

The LC values (LC 50, 90 and 100) of neem powder on *A. aegypti* at different intervals of time are given in Table 3. Indeed, no mortality was recorded at 50% in both of concentrations with neem powder. However, a mortality of 46.90% was obtained at 3 mg/l. This mortality occurred after a relative long time of exposure (120 h) of larvae of *A. aegypti* to the neem powder. Larval mortality at 90% is slightly exceeded with 91.83% at 15 mg/l after 48 h.

Table 2. Mortality (%) of *A. aegypti* treated with 1% formulated neem oil

1% formulated neem oil concentrations (mg/l)	Time of treatment (h)					
	24 h	48 h	72 h	96 h	120 h	144 h
500 ml H_2O (control)	0	4	5	5	6	6
2	39.66 %	65.81	87.63	97.26	98.71	100
4	32.66	74.01	91.41	98.71	98.83	100
6	30	84.43	89.25	93.34	98.71	100
8	50.01	82.59	93.1	95.83	95.83	100
10	64.71	77.08	89.98	93.75	93.75	100
LC 50	8 mg/l (24 h)					

Table 3. Percentage (%) mortality of *A. aegypti* treated with neem powder.

0.3% Neem powder concentrations (mg/l)	Time of treatment (h)				
	24 h	48 h	72 h	96 h	120 h
500 ml H_2O (control)	0	1	1	1	1
3	6	6.12	16.32	46.93	49.98
6	8	8.16	12.12	30.61	32.65
9	4	6.12	8.16	75.51	81.63
12	46	67.34	77.34	97.95	100
15	82	91.83	95.91	100	100
LC 50	3 mg/l (120 h)				

Table 4. Percentage (%) of successful pupation and adult emergence of 4th instars larvae of *A. aegypti*.

Conc. (mg/l)	1% Suneem		Formulated neem oil (F.N.O.)		Conc. (mg/l)	Neem powder (N.P.)	
	Emergence (%)		Emergence (%)			Emergence (%)	
	Pupae	Adults	Pupae	Adults		Pupae	Adults
2	17	3.8	3	22	3	5	2.4
4	7.6	6	21	4	6	6	0
6	5.4	2.6	23	2	9	7	1
8	2.2	1	15	10	12	9	0
10	0	0	20	5	15	9	0
control	12.4	9	25	25	control	25	25

Larvae of *A. aegypti* suffered up to 100% mortalities (LC_{100}) at 12 mg/l after 120 h during treatment. Mortalities of *A. aegypti* are not significantly different with time (ddl: 4; F: -2.604; P = 0.0598 > 5%). However, according to the concentrations, mortalities larvae of *A. Aegypti* treated with neem powder varied significantly (ddl: four; F: 10,094; P = 0.0005 < 5%). In each concentration used, mortalities of *A. aegypti* larvae are significantly important comparing to those of the control (1%) (ddl: 4; F: 7.533; P = 0.0115 < 5%).

Effects on growth and development of *A. aegypti*

Table 4 show the data obtained for the effects of neem products (Suneem, formulated neem oil and neem powder) on growth and development of *A. aegypti* larvae. No adult emergence (0%) was recorded at 10 mg/l for 1% Suneem. However, in this same concentration for formulated neem oil, 5% adults emerged. At 15 mg/l, there was no adult emergence for neem powder. Pupa that emerged at this concentration (15 mg/l), died a few days later. In all treatment with neem powder, no adult emergence was recorded at 50%. The passage of the larvae of *A. aegypti* from nymphs to adults decreased with treatment of 1% Suneem. In comparison with treatment by formulated neem oil, this passage increas-ed. In all of the treatment, majority of the appeared nymphs died, also showing a very white cuticle. Sometimes no nymphs passed into adults and died at this stage (Example: 0% of adults emergence for 10 mg/l Suneem, 12 mg/l and 15 mg/l Neem powder, respectively).

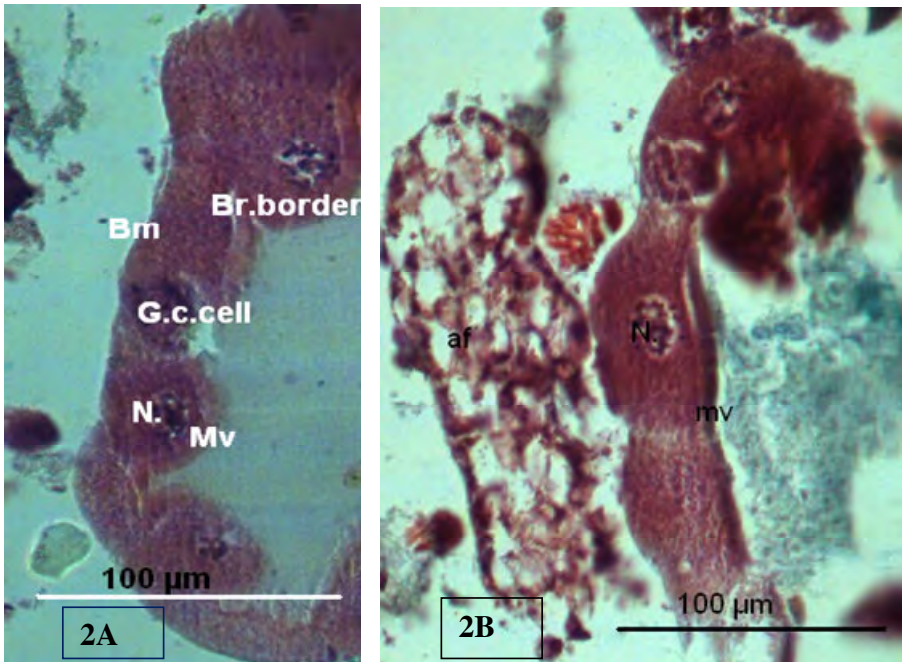

Figure 2. Longitudinal section part in the midgut (Figure 2b) and in the gastric caecum (Ccg). (Figure 2A) of control larva of *A. aegypti* Linneaus 1762. One observes nomals epithelias cells with a nucleus (N) that not affected. These cells were laid on a basal membrane (Bm). Adipose fabric (af) is present. Microvilli (Mv) of bross border (Bb) seem normal.

Comparison between efficacy of neem compounds

The application of "khi-deux" test (X2 test) shows that there was no significant difference in the effects of Suneem and formulated neem oil on *A. aegypti* (ddl = 4; α = 5%; X2 calculated = 5.640 > X2 tabulated = 0.711). These two products have practically similar effects on *A. aegypti* larvae. However, the toxicity of 1% Suneem on *A. aegypti* larvae was significantly different in comparison with the effect of neem powder (ddl = 4; α = 1%; X2 calculated = 39.273 > X2 tabulated = 0.297). The toxicity of 1% formulated neem oil on *A. aegypti* larvae was also significantly different in comparison with the effect of neem powder (ddl = 4; α = 1%; X2 calculated = 60,825 > X2 tabulated = 0.297).

Histopathological effects

The histopathological effects of neem in gastric caeca, stomach regions were studied. The choice of these regions is justified by the fact that they are directly in contact with toxic element (azadirachtin) of neem compounds. The untreated larvae of *A. aegypti* Linneaus, 1762 (control) midgut and gastric caecum showed a well-preserved layer of epithelial cells. The ovoid nuclei are located in the center of the cell. One observes a regularly microvilli border in the midgut and gastric lumens (Figures 2A and B). For the treated larvae of *Aedes aegypti*, the signs of intoxication began on the level of caecum gastric. The histology of *A. aegypti* Linneaus 1762 larvae showed on the level of this region morphologicals and serious damages of the epithelial columnar cells (Figure 3). However, muscles, nuclei and microvilli of brush border appeared very normal as well as adiposis fabric (Figure 3).

The signs of the intoxication continue at the intestinal level precisely in the middle of the gut larvae. The second signs of intoxication consisted in a perturbation of alimentary flow in the alimentary canal (Figure 4). Some cells appear slightly hypertrophied with a perceptible beginning of vacuolization at the apical level. These vacuoles (arrow in Figure 4) invaded the midgut cells. Sometimes, we noted an enlargment of intercellular spaces (Figure 4).

The third signs of intoxication are perceptible in the posterior part of the gut of *A. aegypti* larvae. Epithelial cells of the intestine start to burst and we noted a cytoplasmic rejection of cells material that mixed with food column (Figure 4).

On the level of the anterior and posterior intestine, some cells degenerated and showed beaches of lysis (Figure 3). These cells are very advanced in their infection.

DISCUSSION

The neem products show remarkable bioactivity against *A. aegypti* larvae. Azmi et al. (1998) report LC_{50} of Neem

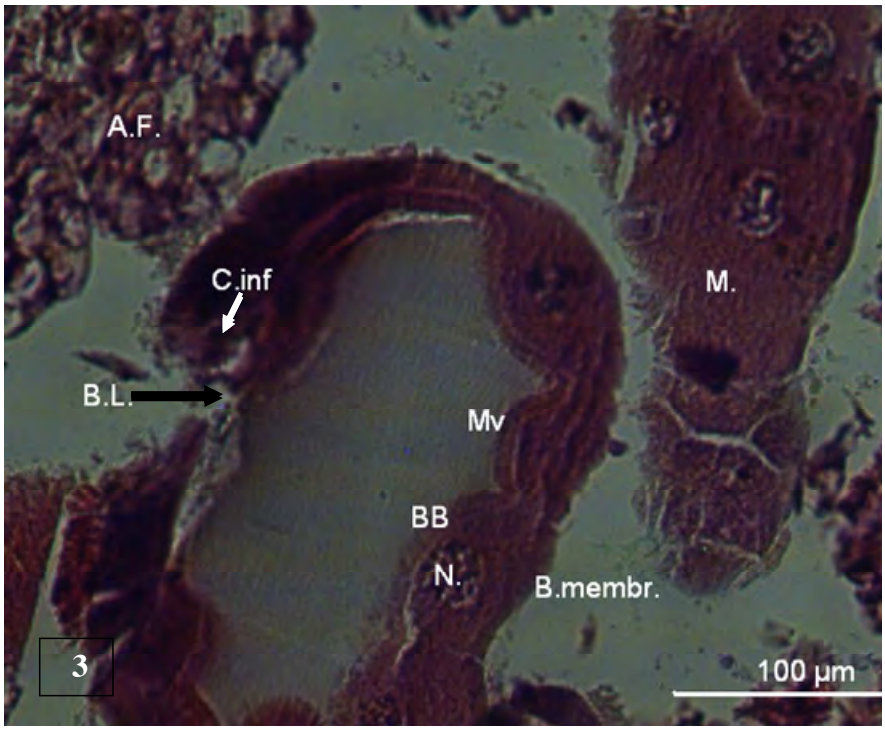

Figure 3. Longitudinal section part of gastric caecum of *A. aegypti* Linneaus 1762. This section of larvae treated with neem products showed a morphological lesions of gastric epithelial cells (GEC) with beach lysis (BL). Nuclei (N), muscles (M), adipose fabric (AF) are not affected and seem normal. Microvilli of Brush border (BB) are not affected (B. membr, basal membrane; and C.inf, cells infected).

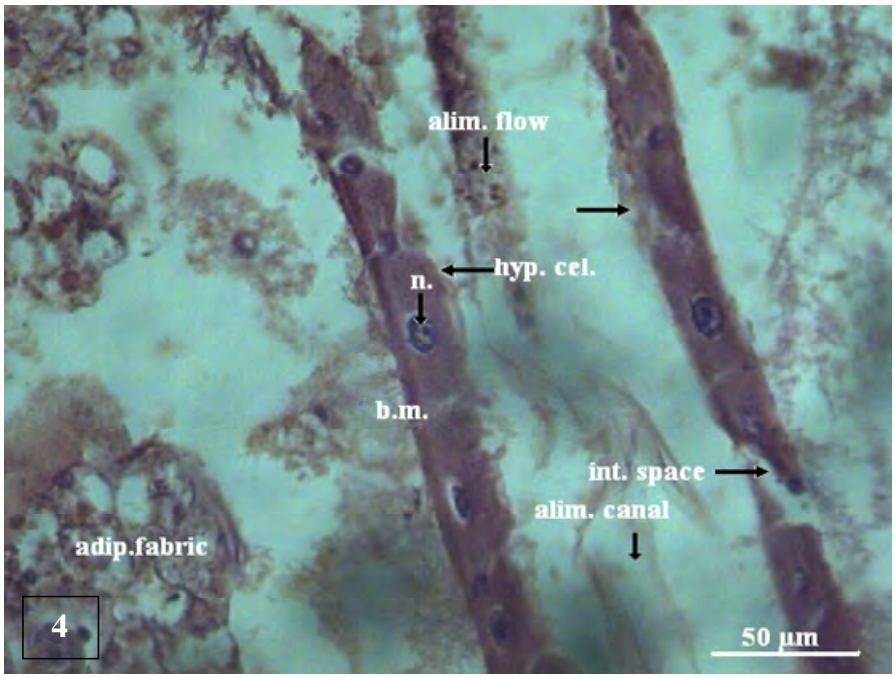

Figure 4. Longitudinal section part of the midgut of 4th instars larvae of *A. aegypti*. The midgut cells are slightly hypertrophied (arrow; hyp. cel.). Sometimes, we noted an enlargment of intercellular spaces (int. space) and cells lysis. The evident sign consisted here to an absence of alimentary flow (Alim. flow) in the alimentary canal (Alim.canal). (BB, brush border; adip. fabric., adiposis fabric; and b.m., basal membrane).

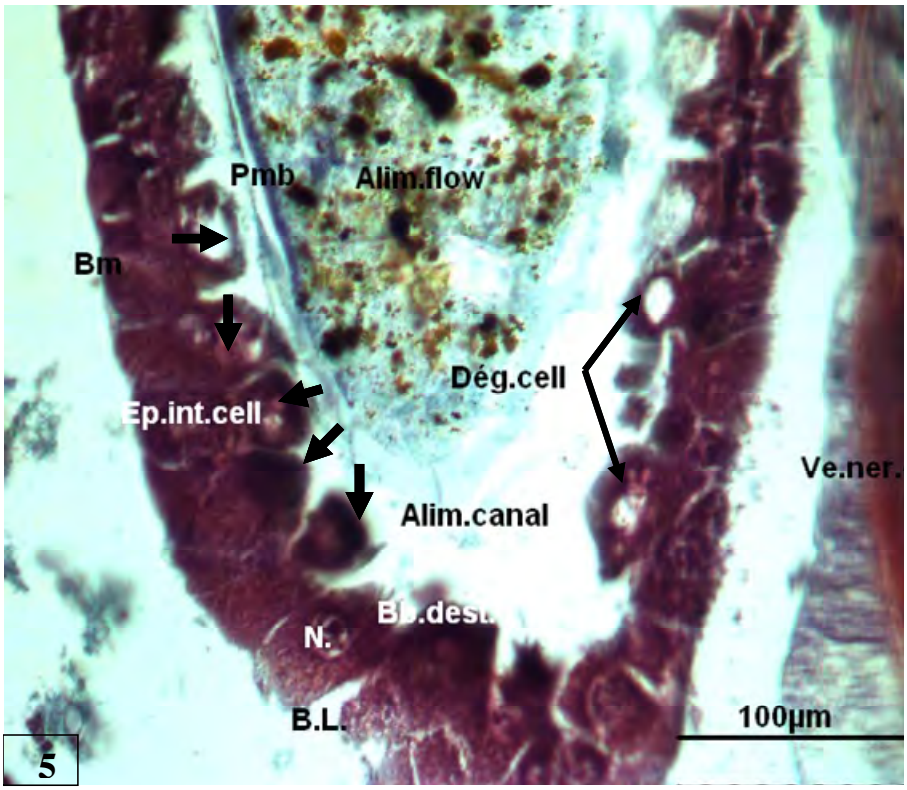

Figure 5. Longitudinal section in posterior part of the gut of *A. aegypti* Linneaus 1762 showed that some cells appeared to be degenerated (Dég. cell and arrows). Epithelial intestinal cells (Ep.int.cell) showed a beach of lysis (BL). Cells are in an advanced stage of infection (head of arrows). The ventral nerve chain (VNC) appeared to be normal. Bross border (Bb.destr.), microvilli (Mv) and peritrophic membrane (Pmb) are destroyed. Nuclear (N), alimentary flow (Alim.flow) is present in alimentary canal (Alim.canal). Bm = Basal membrane.

Leaves Extracts (N.L.X.) values against late third instar larvae of *Culex fatigans* Wild Strain at 390 ppm. In the present finding related in this article, the LC_{50} values of neem products were 2 mg/l in 24 h for 1% Suneem, 8 mg/l in 24 h for formulated neem oil (F.N.O.) and 3 mg/l in 96 h for neem powder. This LC_{50} of neem products found in this study was lower compared with the results of Azmi et al. (1998). The neem products that Azmi et al. (1998) used in their study are more effective than those used in the present study. This difference can also be due to insects species used, environment of the bioassays and the mode of treatment. Wandscheer et al. (2004) study on different ethanolic extracts of *Melia azedarach* and *A. indica* against the dengue mosquitoes *A. aegypti* Linneaus 1762 in the laboratory observed LC_{50} values at 25 and 30°C as 0.166 and 0.152 g%, respectively, for *M. azedarach* and 0.044 and 0.063 g%, respectively, for *A. indica*. In this present investigation, the LC_{50} for 1% Suneem, formulated neem oil and neem powder were 2, 8 and 3 mg/l. These results appear comparable with those obtained by Wandscheer et al. (2004). However, neem used in this work is slightly more effective than the ethanolic extracts of *M. azedarach* and *A. indica*.

Naqvi et al. (1991) in determining the toxicity of crude neem extract (NFD) on *A. aegypti* (PCSIR strain), obtained LC_{50} as 0.58 ppm of NFD, 0.625 ppm for nimocinolide and 0.47 ppm for isonimocinolide. In our present results, the 1% Suneem, formulated neem oil and neem powder are slightly more toxic than crude neem extract (NFD), nimocinolide and isonimocinolide. This difference could be due to morphology and physiology of insects species used, change of environment of the bioassays and mode of treatment. Amorose (1995) reports LC_{50} values of neem oil and deoiled neem cake against 3^{rd} and 4^{th} larval instars of *Culex quinquefasciatus* Say 1823 at 0.99 and 1.20, 0.55 and 0.72 ppm respectively. The toxicity of 1% Suneem, formulated neem oil and neem powder is more evident compared to the toxicity of neem oil and deoiled neem used by Amorose (1995) against mosquitoes larvae.

El hag et al.(1999) reported that no further larval development of *Culex pipiens* took place beyond the second instars larvae at concentrations of 400 ppm and above in the *Rhazya stricta* methanol extract, and only 3.3% successful pupation occurs at 200 ppm. In addition,

no adult emergency were observed in any of the two *R. stricta* extracts except for 3.3% in the ether extract at 400 ppm. However, in our study, no nymphs and adults emergence of *A. aegypti* Linnaeus 1762 took place at concentration of 10 mg/l for 1% Suneem; all larvae have been killed. Therefore, 1% Suneem is more effective than *R. stricta* methanol extract. El hag et al. (1999) also reported that all three materials (*A. indica*, *R. stricta*, *Syzygium aromaticum*) tested conferred significant negative influences on larval development to varying degrees, consequently reducing pupation and inhibiting adult emergence. In our study, no nymphs and adults emergence of *A. aegypti* occured at 10 mg/l for 1% Suneem. Moreover, dead nymphs were covered with a very white cuticle. This whiteness of the cuticle can be due to his non-sclerotisation.

Histopathological effects of neem products are presented in Figures 2, 3, 4 and 5. Neem compounds showed remarkable effects on caecum and gut of *A. aegypti* larvae. Karch and Coz (1983) and Kallen et al. (1965) reported that the ingestion of *Bacillus sphaericus* 1593-4 by the larvae of *Culex pipiens* and by the larvae of *Culex tarsalis* revealed a perturbation of alimentary flow in the alimentary canal, a hypertrophy or swelling of cells in the posterior part of the gut. Some vacuoles invaded the midgut cells. In an advance stage of infection of the cells, lysosomes appeared in the apical part. A bursting of the cell occurred, followed by a rejection of the cytoplasmic material into the ectoperitrophic space. In our investigation, we reported a morphological and serious damage in epithelial columnar cells and a perturbation of alimentary flow in alimentary canal. In posterior part of gut, cells appear slightly hypertrophied with a perceptible beginning of vacuolisation. Cells start to burst with cytoplasmic rejection of cells material. Our results corroborate the observations of Karch and Coz (1983) and Kallen et al. (1965). We note that a morphological evolution of the intoxication differ from one region to another. Indeed, the cellular damage as well as the degree of intoxication is not the same on all levels in the gastric caeca and the intestinal regions. This fact seems linked to the difference of morphological and physiological cells in oesophagus, gastric and intestine regions. Koua et al. (1998) reported that after the treatment of *Anopheles gambiae* with aqueous extract of *Persea americana*, the larvae suffer important damages in the midgut with hypertrophic cells. Then most of the cells lyse, with a rejection of cytoplasmic material towards the lumen gut, between the peritrophic membran and the midgut epithelium. Almost, total cell degeneration is observed. In our investigation, we obtain the same results. Cavados et al. (2004) revealed, in these study of *Simulium pertinax* larvae treated with *Bacillus thuringiensis* Serovar *israelensis*, a serious damage of the epithelial columnar cells. The most characteristic effects were midgut columnar cells vacuolization, microvilli damages, epithelium cell contents passing into the midgut lumen and finally the cell death. Our observations are also in agreement with these results.

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REFERENCES

Abbot WS (1925). A method of computing the effectiveness of an insecticide. J. Econ. Entomol., 18: 265-267.

Aliero BL (2003). Larvaecidal effects of aqueous extracts of *Azadirachta indica* (neem) on the larvae of *Anopheles* mosquito, Afr. J. Biotechnol. 2(9): 325-327.

Amorose T (1995). Larvicidal efficacy of neem (*Azadirachta indica*, A Juss) oil and defatted cake on *Culex quinquefasciatus* Say. Geobios (Judhpur) 22: 168-169.

Azmi AM, Naqvi SNH, Ahmad I, Tabassum R, Anbreen B (1998). Toxicity of Neem Leaves Extract (NLX) Compared with Malathion (57 E.C.) against late 3rd instars of *Culex fatigans* (Wild Strain) by WHO method. Tr. J. Zool., 22: 213-218.

Cavados CFG, Majerowicz S, Chaves JQ, Araùjo-Coutinho CJPC, Rabinovitch L (2004). Histopathological and Ultrastructural effects of δ-endotoxins of *Bacillus thuringiensis* Serovar *israelensis* in the midgut of *Simulium pertinax* larvae (Diptera, Simuliidae). *Mem Inst Oswaldo Cruz*, Rio de Janeiro, 99(5): 493-498.

El Hag EA, El Nadi AH, Zaitoon AA (1999). Toxic and growth retarding effects of three plant extracts on *Culex pipiens* larvae (Diptera : Culicidae). Phytother. Res., 13: 388-392.

Kallen WR, Clark TB, Lindegren JE, Ho CB, Rogoff MH, Singer S (1965). *Bacillus sphaericus* Neide as a pathogen of mosquitoes. J. Invert. Pathol., 7: 442-448.

Karch S, Coz J (1983). Histopathologie de *Culex pipiens* Linné (Diptera, Culicidae) soumis à l'activité larvicide de *Bacillus sphaericus* 1593-4, Cah. O.R.S.T.O.M., Sér. Ent. Méd. Parasitol., vol. XXI, n° 4 : 225-230.

Konan YL, Koffi AA, Doannio JMC, Darriet F (2003). Résistance de *Culex quinquefasciatus* Say, 1823 à la deltaméthrine et l'utilisation de la moustiquaire imprégnée en milieu urbain de Bouaké, Côte-d'ivoire. Bull. Soc. Pathol. Exot. 96(2): 128-129.

Koua HK, Han SH, d' Almeida MA (1998). Histopathology of *Anopheles gambiae s.l.* Giles, 1902 (*Diptera*, *Culicidae*) subjected to the larvicidal activity of the aqueous extract of *Persea americana* Miller, 1869 (Lauraceae). Bull. Soc. Pathol. Exot. 91(3): 252-256.

Martoja R, Martoja M (1967). Initiation aux Techniques de L'histologie animale. Masson ed. Paris, pp. 345-368.

Naganishi CK (1975). Structure of the insect antifeedant azadirachtin. In: Recent advances in photochemistry, VC Runckles (ed.), Plenum, New York, 5: 283-298.

Naqvi SNH, Ahmed SO, Mohammed FA (1991). Toxicity and IGR (Insect Growth Regulating) effect of two neem products against *Aedes aegypti* (PCSIR Strain). Pak. J. Pharm. Sci., 4(1): 71-76.

Salehzadeh A, Akhkha A, Cushley W, Adams RLP, Kusel JR, Strang RHC (2003). The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells. Insect Biochem. Mol. Biol. 33: 681-689.

Schumuthere H, Ascher KRS, Rembold H (1981). Natural pesticids from the neem tree. Proc.1st int. Neem Conf. Rottach – Egem: 294.

Scott M, Kaushik NK (2000). The Toxicity of a Neem Insecticide to populations of *Culicidae* and other aquatic invertebrates as assessed in in situ microcosms. Arch. Environ. Contam. Toxicol., 39: 329-336.

Sivagnaname S, Kalyanasundaram M (2004). Laboratory evaluation of Methanolic extract of *Atlanta monophylla* (Family: Rutaceae) against immature stage of mosquitoes and non-target organisms. Mem. Inst Oswaldo Cruz, 99(1): 115-118.

Tianyun S, Mulla MS (1999). Oviposition bioassay responses of *Culex quinquefasciatus* Say, 1823 and *Culex tarsalis* to neem products containing azadirachtin. Entomol. Exp. Appl., 91: 337-345.

Wandscheer CB, Duque JE, Da Silva MAN, Fukuyama Y, Wohlke JL, Adelmann J, Fontana JD (2004). Larvaecidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the Dengue mosquito *Aedes aegypti*. Toxicon, 44(8): 829-835.

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Full Length Research Article

Knockdown (kdr) mutation in the resistance of Culex quinquefasciatus mosquitoes to Suneem 1% (*Azadirachta indica, A. Juss*)

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Abstract

Susceptibility levels of *Culex quinquefasciatus* to Suneem 1% have been determined. Concentrations of 2 mg/l, 4 mg/l, 6 mg/l, 8 mg/l, 10 mg/l, 12 mg/l, 14 mg/l, 16 mg/l, 18 mg/l and 20 mg/l were used to control larval stages. Some of them i.e. 9mg/l, 12mg/l, 14mg/l and 18mg/l resulted in about 50 to 90% mortality of young larval stages (1 and 2) after 24 hours and 48 hours respectively. For older larval stages (3 and 4), concentrations higher than 20 mg/l caused 50 to 90% mortality. Even though Suneem 1% seems to be very effective, some *Culex quinquefasciatus* larvae show resistance. Indeed, after amplification of the isolated DNA, the electrophoresis revealed 1200 and 1400 bp of the amplicons for the natural population (DsenCqG0) and for generation 1 (DSenCqG1) of *Culex quinquefasciatus*. For the 5th generation (DSenCqG5), electrophoresis has shown an amplification of kdr gene between 100 and 200 bp. Comparing the sequences of DSenCqG0 and DSenCqG5 of *Culex quinquefasciatus*, differences were noted in the bases at the positions G1A, C2A, G3C, T4A, G5C, T6A, G7A, T8A, T9A, T11C, T12C, T14C, T15A, T16A, T17A. Differences were also noticed at positions G501A, G502A, G503A, C504A, C505A, T506A, T510G, T511G, T512A and at positions T569A, T571G, T572A, T573A, T574C, C575A, C577A, T578C, T579C and C582A. To control mosquitoes of *Culex quinquefasciatus* and others, genus Suneem 1% proved to be effective but resistance can be a major obstacle.

Key words: Treatment, *Culex quinquefasciatus*, Suneem 1%, Mortality, resistance

INTRODUCTION

Diseases like malaria, yellow fever, dengue fever and filariasis are common in the cities of African countries one of which is Senegal. Such diseases are caused by the proliferation of mosquito's larvae in natural breeding sites. In order to fight against mosquitoes in Senegal, chemical insecticides are used on larvae, pupae and adults. Many treatments with chemical insecticides have given larval stages that have the ability to tolerate toxic doses. These are mutant larval stages which have physiological and enzymatic equipment that allow them to survive to lethal doses of chemical insecticides.

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Resistant species of *A. gambiae* (*A. saccharovi*, *A. stephensi* and *A. Culicifacies sundarcus*) were noted with the use of DDT (Dichloro-Diphényl-Trichloroéthane) and Dieldrin (Hamon and Garrett-Jones, 1963). *Culex tarsalis* has developed resistance to Malathion in California. In 1960, a similar resistance to Malathion was observed in *Culex pipiens fatigans* Wiedeman in Cameroon (Mouchet *et al*., 1960). *Aedes nigromaculis* (Ludlow) has developed resistance to organophosphates in California (Lewallen et al., 1963). Also, records include laboratory induced resistance of *Aedes aegypti* to Malathion (Abeni and Brown, 1960).

Resistance began to develop after repetitive treatments of mosquito larvae with insecticidal products of biological origin, such as neem (*Azadirachta indica* A. Juss). This observation was made after applying several treatments with neem products to mosquito larvae. Natural population of mosquito larval stages (DSenCqG0) have survived to different neem concentrations. These observations were made during the studies of Seye *et al*. (2006a,b), Ndione *et al*. (2006, 2007). In this context, this research aims to study mosquito's resistance to neem products like Suneem 1%.

MATERIALS AND METHODS

Culex quinquefasciatus strains

Three differents strains of *Culex quinquefasciatus* were used in this study, there are as follows:

- DSenCqG0 strains (Dakar, Senegal, *Culex quinquefasciatus*, generation zero). The Larval stages of *Culex quinquefasciatus* were collected from mosquito's habitats in the Cheikh Anta Diop university campus of Dakar and reared with bread powder in laboratory conditions 27°C ± 5°C of temperature, under a relative humidity of 82%±10% and a photoperiod of 12:12 (L:D) hours.
- DSenCqG1 strains (Dakar, Senegal *Culex quinquefasciatus*, generation one) that have been treated and killed after 24 hours of treatment with 1% Suneem.
- DSenCqG5 strains (Dakar, Senegal, *Culex quinquefasciatus*, generation five) that have been treated with Suneem 1% for five (5) generations after collection.

The bioinsecticidal product: Suneem 1 % EC (Emulsiable Concentrations)

The bioinsecticidal product used in this study is Suneem 1%. It is a product formulated by azadirachtin and a solvent (Tetrahydrofurfuryl alcohol: THFA), produced by a Senegalese chemical industry (Senchim) situated in Rufisque, Dakar (Senegal).

Suneem 1% susceptibility bioassay

Culex quinquefasciatus collected from the natural breeding sites and from the artificial sites of the Department of Animal Biology of Cheikh Anta Diop University of Dakar, represent DSenCqG0 strain. To have DSenCqG5 strain, DSenCqG0 strain of *Culex quinquefasciatus* were firstly immersed in 500 ml of distilled water and then treated with the concentrations of Suneem 1% (2 mg/l, 4 mg/l, 6 mg/l, 8 mg/l, 10 mg/l, 12 mg/l, 14 mg/l, 16 mg/l, 18 mg/l and 20 mg/l) in laboratory conditions (temperature ~ 27°C ± 5°C and a relative humidity ~ 82°C ± 10°C). The Suneem 1% concentration of each experimental variant was sufficient to kill 50 and 90% (CL 50, CL 90) in 1 and 2 larval stages after 24 hours. To select DSenCqG1 strains, DSenCqG0 strains of *Culex quinquefasciatus* were treated with different concentrations (2 mg/l, 4 mg/l, 6 mg/l, 8 mg/l, 10 mg/l, 12 mg/l, 14 mg/l, 16 mg/l, 18 mg/l and 20 mg/l) after 24 hours.

DNA isolation

All *Culex quinquefasciatus* mosquito strains were dried at 75°C before DNA extracation. Genomic DNA from 1% Suneem survivors (DSenCqG5), 1% Suneem dead (DSenCqG0) and natural population (DSenCqG1) was extracted using the phenol-chloroform method as described. Samples were placed in 1.5 ml Eppendorf tubes containing 300µl lysis buffer (50Mm Tris- HCl, pH = 8, 1% SDS, 25 Mm EDTA) and 20 µl of K proteinase 20 mg/ml and the tube was incubated in a water bath at 65ºC for 60 mins. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) has been added and the tubes were gently shaken to mix the phases. The tubes were centrifuged at 12000 rpm for 5 mins to separate the phases.

The aqueous phase was moved to new tubes and an equal volume of chloroform was added. The DNA was precipitated in 1/10 volume of 3M sodium acetate pH = 5.2 and 2 volumes of 100% ethanol stored at - 20°C. The solution is 10 mins centrifuged at 14,000 rpm and the DNA pellet washed with 1ml of 70% ethanol. The pellet was then dried, followed by an elution in ultrapure water (Promega).

Amplifications of Sodium Channel gene of *Culex quinquefasciatus*

PCR was performed to detect kdr mutation following the Martine-Torres *et al.* (1999) protocol, with minor modifications to reagent concentrations. The sodium channel gene contains the kdr mutation. Two rounds of PCR reactions were performed in 20 µl final reaction volume, containing 1X PCR buffer, 10 mM of dNTP, 50 mM MgCl2, 2.5 U Taq polymerase, 1 µl of DNA template and a pair of primers, KDR S1 (Forward)

Table 1. Susceptibility of *Culex quinquefasciatus* larvae to 1% Suneem

Larval stages	Treatment duration	n	LC_{50}	LC_{90}	RR_{50}	RR_{90}
Cx quinquefasciatus (1 and 2 larval stages)	24 hours	100	12 mg/l	18 mg/l	1,33	2
	48 hours	100	9 mg/l	14 mg/l	1	1,55
Cx quinquefasciatus (3 and 4 larval stages)	24 hours	100	> 20 mg/l	> 20 mg/l	1,44	1
	48 hours	100	14 mg/l	> 20 mg/l	1	1

Key: n: number of mosquitoes treated
RR: were calculated as the ratio of susceptible strains divided by the ratio of non susceptible (or resistant) strain

5'CTTACTCATTTCCATCATGG3'and KDR AS1 (Reverse) 5'GACAAAAGCAAGGCTAAGAAAAGG 3'. For PCR reaction, an Thermo PX2 thermal cycler was used and the amplification stepts: one step 95°C for 5 mins, followed by 40 cycles denaturation 94°C for 30s, annealing 55°C for 30s, extension 72°C for 1 min, followed by final extension of 72°C for 10 mins. The second round of PCR, containing 1 µl of the first PCR solution, a pair of primers KDR ALS1 (Forward) 5'GCGTTAGGTAATCTGACGTTTGTGC3' and KDR AS1 (Reverse) 5'GACAAAAGCAAGGCTAAGAAAAGG3' was conducted under the same reaction conditions described above, except that 38 cycles and a 58°C of annealing temperature were used.

Electrophoresis

To obtain 2% agarose gel, 2 g of agarose was mixed in 100 ml of 0.5 x TBE and then dissolved in a microwave oven for 2 mins. 5 µl of ethidium bromide (10 mg/ml) were added in the agarose gel. The polymerized dried gel was transferred into an electrophoresys tray containing 0.5 x TBE buffer as electrolyte. To make the DNA traces visible, the amplicons were mixed with 5 µl bromophenol blue. The migration was carried out under a current of 150 Volts for 1 hour using a generator "Electrophoresis Power Supply- EPS 301 (Amersham Pharmacia Biotech.). After migration, bands were visualized and analyzed using a PhotoDoc system UVP. The size of the amplified products was estimated based on the presence of 100 bp molecular weight marker with bands of known molecular weights (100 bp DNA Ladder, Ready-Load TM Invitrogen, Cat. No. 10380 - 012). The targeted regions are: 293 bp control, 195 bp resistant allele and 137 bp susceptible allele.

Amplicons purification

The selected amplicons were purified using a Wizard SV Gel and PCR clean-up system (Promega) kit, according to the producer protocol.

DNA sequencing reaction preparation

The sequencing reaction was prepared in 0.2 ml reaction volume, using a DTCS Quick Start Master Mix (Beckman Coulter). All reagents were kept on ice while preparing and sequencing reactions were added in the order listed below. dH20: 0 - 9, 5 µl, the DNA template (0, 5 – 100 µl), sequencing primers 1,6 µM - 2µl, reaction master mix: 8,0 µl.
It should be noted that the reaction components were thoroughly mixed. The liquid is bound to the bottom of the tube by a brief centrifugation prior to sequencing. This reaction mixture was subjected to a sequencing cycle in the sequencer: 96°C for 20 s, 50°C for 20s and 60°C (4 mins) during 30 cycles at 4°C.

Sequencing reaction Purification

The sequencing unincorporated ddNTP's, primers and other sequencing reaction components are removed using fresh prepared glycogen solution: 3M NaAc (µl): 2µ l, 100 mM Na-EDTA: 2 µ l, 20 mg/ml glycogen (1µl). 5 µl of glycogen solution was added in new properly labeled 0.2 ml tubes, the sequencing reaction products were transferred and mixed thoroughly. 60 µl of (V/V) ethanol/water -20°C cold mixture were added and mixed again. The tubes were centrifuged at 14,000 rpm 4°C for 15 mins. The supernatant was carefully removed and subsequently the pellet dried for 10 mins and resuspended in 40 cl of samples loading solution provided with the kit.

Sequencing

Sequencing of purified fragments was performed with a Beckman Coulter CEQ 8000 genetic analyser. The sequence were analysed and aligned using MEGA 6 Software (Tamura *et al.*, 2013).

RESULTS

Susceptibility of *Culex quinquefasciatus* to Suneem 1%

Table 1 gives the values of CL50 and CL90 of the young larval stages (1 and 2) and olders larval stages (3 and 4) of *Culex quinquefasciatus* after 24 and 48 hours. Resistance Ratio (RR) at 50% of *Culex quinquefasciatus* to Suneem 1% are 1.33 and 1 respectively and those at

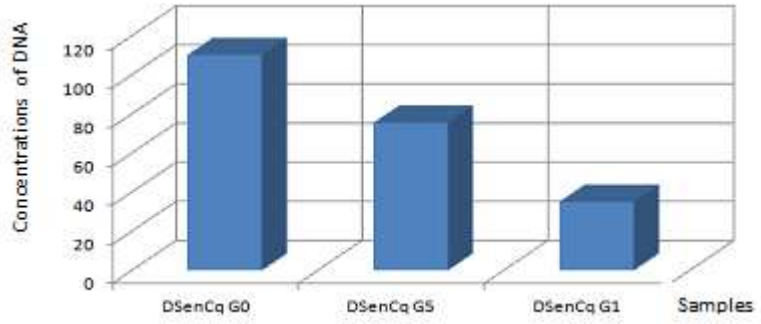

Figure 1. DNA concentrations isolated by phenol-chloroform method from the three samples of *Culex quinquefasciatus*

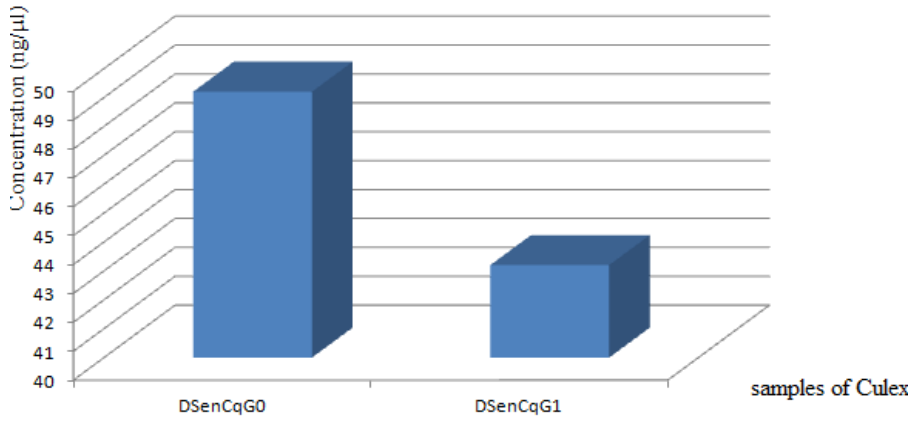

Figure 2. PCR Amplicons concentrations for *Culex quinquefasciatus*

90% are 2 and 1.55 for young instars larvae. On the other hand, for young and old instars larvae, the rates of resistance at 50% and 90% are 1.44 and 1 respectively. It was noted that the concentration of the DNA samples of *Culex quinquefasciatus* of natural population (DSenCq G0) is 105 ng/µ l, for the populations of generation 5 (G5 DSenCq) is 70 ng/µl and for the population of generation 1 (G1 DSenCq) i s 30 ng/µl (Figure 1).

This extraction method gave a DNA concentration of the *Culex quinquefasciatus* generation G0 (DSenCqG0) greater than 40ng/µl and 80ng/µl for G5 (DSenCqG5) and G1 (DSenCqG1) generations. Figure 2 shows the *Culex quinquefasciatus* natural population (DSenCqG0) and generation 1 (DSenCqG1) PCR amplicons concentration. The spectrophotometric DNA amplicons analysis shows a concentration of 48.5 ng/µl for the natural population of *Culex quinquefasciatus* (DSenCqG0) and 42.5 ng/µl the amplicons concentration for the generation 1 (DSenCqG1) equal to. The amplicons agarose gel electrophoreses have between 1200 bp and 1400 bp length for the natural population of *Culex quinquefasciatus* (DsenCqG0) and also for generation 1 (DSenCqG1). But for the generation 5 (DSenCqG5), electrophoresis shows an amplification of kdr gene between 100 and 200 bp (Figure 3). Figure 4 shows a "susceptible" and "kdr mutation" genotypes obtained.

By comparing the two sequences in Tables 2, 3 and 4 (Sequence of KDR gene of natural population of *Culex quinquefasciatus*:DSenCqG0 and Sequence of KDR gene of treated population of *Culex quinquefasciatus*: DSenCqG5), differences were noted in the bases at positions G1A, C2A, G3C, T4A, G5C, T6A, G7A, T8A, T9A, T11C, T12C, T14C, T15A, T16A, T17A. Differences can be noticed at positions G501A, G502A, G503A, C504A, C505A, T506A, T510G, T511G and T512A and also at T569A, T571G, T572A, T573A, T574C, C575A, C577A, T578C, T579C and C582A. The differences in the bases are shown in red colour.

DISCUSSION

This study aim to analyse the resistance of *Culex quinquefasciatus* mosquito to Suneem 1%. It showed a

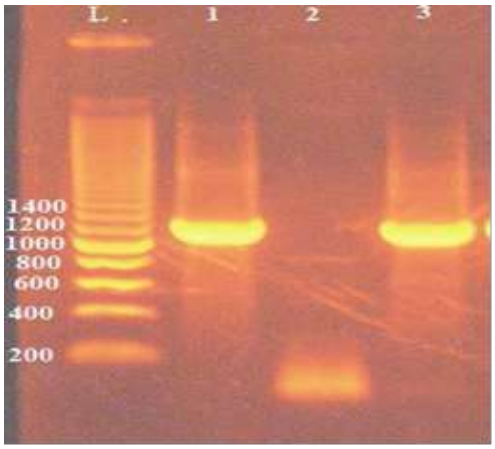

Figure 3. Amplications of kdr gene for the three samples of *Culex quinquefasciatus* showed by electrophoresis on agarose gel (Promega®). L.= step ladder (100bp molecular weight marker), 1= DSenCqG0, 2= DSenCqG5 and 3 = DSenCqG1

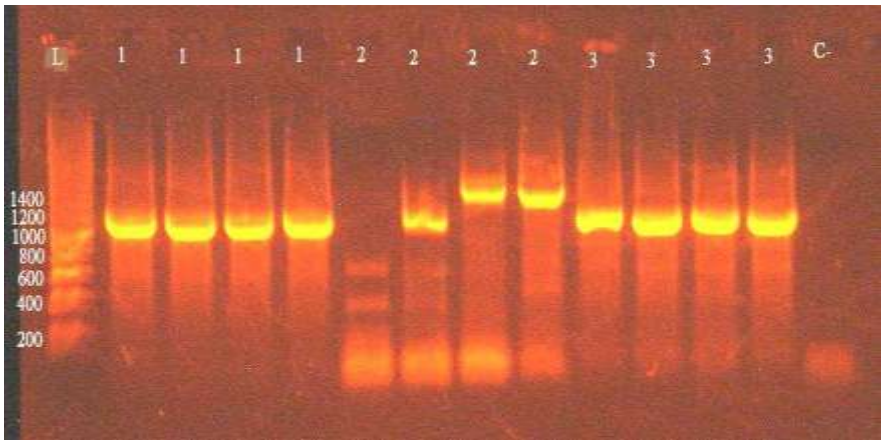

Figure 4. Amplicons of kdr gene of the three samples (five individuals for each sample) of *Culex quinquefasciatus* showed by electrophoresis on agarose gel (Promega®). L.= ladder, 1= DSenCqG0, 2= DSenCqG5 and 3 = DSenCqG1.
Lanes L and C- : DNA ladder and negative control **Lane 1**: sus/sus is susceptible genotype
Lane 2: kdr/kdr is kdr mutation genotype **Lane 3**: sus/sus is susceptible genotype

Table 2. Sequences (10bp to 40bp) of « natural » and « treated » population (DSenCqG5) of Culex quinquefasciatus and sequence reference

Sequence of KDR gene of natural population of *Culex quinquefasciatus* (DSenCqG0)
Sequence of KDR gene of treated population of *Culex quinquefasciatus* (DSenCqG5)**
G A C T G A A T T T C C C T A T A C A C T A C A G T G G C C A A G A A A A A T G*
A C G A C T G A A T T T C C T A T C A C T A C A G T G G C C A A G A A A A A T G**
10 **20** **30** **40**
reference sequence of *Culex quinquefasciatus*
G C G T G T G T T T T T C T T T T C A C T A C A G T G G C C A A G A A A A A T G

Table 3. Sequences (490bp to 520bp) of « natural » and « treated » population (DSenCqG5) of Culex quinquefasciatus and sequence reference

Sequence of KDR gene of natural population of *Culex quinquefasciatus* (DSenCqG0)
Sequence of KDR gene of treated population of *Culex quinquefasciatus* (DSenCqG5)**
A A A T T T T G G C T G A T T T G G G C G A G A C A T T T T G T A C T T G T T G*
A A A T T T T G G C T G A T T T G G G C A G A C A T T T T G T A A C T T G T T G**
490 **500** **510** **520**
reference sequence of *Culex quinquefasciatus*
A A A T T T T G G C T G A T T T G G G C G G G C C T T T T T T T A C T T G T T G

Table 4. Sequences (570bp to 600bp) of « natural » and « treated » population (DSenCqG5) of Culex quinquefasciatus and sequence reference

Sequence of KDR gene of natural population of *Culex quinquefasciatus* (DSenCqG0)
Sequence of KDR gene of treated population of *Culex quinquefasciatus* (DSenCqG5) **
T A G T A A A A T T T A T C A A C T C C C A A T T G C T T T T T T A T T T G C C*
T A G T A A A A A T G T A T C A A C T C C C A T T G C T T T T T T A T T T G C C**
570 **580** **590** **600**
reference sequence of *Culex quinquefasciatus*
T A G T A A A A T T T T T T C A C T T C C C A T T G C T T T T T T A T T T G C C

CL 50 and CL 90 (lethal concentrations 50 and 90) of 9 mg/l and 14 mg/l respectively after 48 hours for young larval stages (1 and 2). And for the older larval stages (3 and 4) of *Culex quinquefasciatus*, the concentrations which result a mortality of 50% and 90% are respectively 14 mg/l and 20 mg/l. The CL 50 obtained in this study has showed a significant difference with the CL50 obtained by Hougard *et al*., 1983. Indeed, Hougard *et al*.., 1983 obtained 0,10 mg/l (CL 50) by treating young larval stages of *Culex quinquefasciatus* using *Bacillus thuringensis ser. Israelensis*. The CL 50 obtained in this study is significantly higher than the CL 50 obtained by Hougard *et al*. (1983).

So the biopesticide of *Bacillus thuringensis ser. Israelensis* is more effective than the biopesticide based on neem. However, the CL50 (9 mg/l) obtained in this study with young larval stages of *Culex quinquefasciatus* are similar to those obtained by Ndione *et al*., 2007, treating *Aedes aegypti* by Suneem 1% have obtained 8 mg/l. The study also examined DNA sequences and especially the kdr mutation who determined the resistance of mosquito *Cx. quinquefasciatus* to Suneem 1% (Azadirachta indica, A. Juss). We found in this study a mutation of the kdr gene after treatment by Suneem 1% of the larval stages of *Cx. quinquefasciatus* after five generations. Determining insensitivity or resistance in *Culex quinquefasciatus* Suneem 1% (local biopesticide) electrophoresis revealed a amplification of kdr gene sodium channel voltage-gated between 1200 bp and 1400 bp length for the natural population of *Culex quinquefasciatus* (DsenCqG0) and also for generation 1 (DSenCqG1). But for the generation 5 (DSenCqG5), electrophoresis shows an amplification of kdr gene between 100 and 200 bp. These results obtained with *Culex quinquefasciatus* in this study are not similar with those of Saavedra-Rodriguez *et al.* (2007). They found amplification of the kdr gene voltage- dependent sodium channel to 125 bp for *Aedes aegypti* mosquitoes resistant to pyrethroids. The difference between the results of Saavedra-Rodriguez *and al.* (2007) and those of this study can be explained by the difference of the species.

Furthermore, the nucleotide sequences obtained from natural populations (*Culex quinquefasciatus* untreated Suneem by 1%) (DSenCqG0) and those treated and dead after 24 hours showed differences in the sequence of the bases at positions 17 and positions 602-605. Their analysis showed a result of a mutation of a base substitution of thymine for adenine one another. This shift will cause the replacement of a leucine Thymine Thymine Adenine (TTA) with phenylalanine Thymine Thymine Thymine (TTT). This mutation could cause insensitivity or even a resistance of *Culex quinquefasciatus* to Suneem 1%. These results are similar to the work of Singh et al. (2011). Indeed, the work of Singh *et al.* (2011) revealed the presence of two nonsynonymous mutations alternatives between (T and C) and (A and T) respectively at the 2nd and 3rd codon position. This is the origin of mutation of a Leucine (TTA) by a Serine (TCA) and by a phenylalanine (TTT).

This mutation is the cause of resistance which occurred after changing the voltage-dependent sodium channel. This insensitivity or resistance of the larvae of *Culex quinquefasciatus* treated could be explained by a selection pressure of pesticides used in treatment

campaigns mosquitoes. The interpretation of the results of this study found is base to those of Sinègre *et al*., 1976). According to Sinègre *et al*. (1976), the insensitivity or resistance of Culicidae populations is due to the selection pressure of agricultural pesticides that *Culex pipiens* are subjected in suburban, urban and rural habitats. Furthermore, the voltage-dependent sodium channel is the first site where most especially insecticides biopesticides act (Narahashi, 1996; Sattelle and Yamamoto, 1998.

Indeed, their results revealed a change in the conformation of the voltage-gated sodium channel of *Culex quinquefasciatus* after 5 generations (F5). Haubruge and Amichot (1998) in their study developed the main mechanisms responsible for resistance including behavioral resistance associated with the mobility of the insect, the physiological changes associated with the penetration kinetics, sequestration and excretion of insecticide, and that associated with activation biochemical detoxification systems.

The results of this study are consistent with a mechanism described by Haubruge and Amichot (1998) i.e. the physiological mechanism of resistance associated with the change in the kinetics of penetration. In this mechanism most often the kdr gene can be over expressed and synthesized proteins prevent insecticide to kill mosquitoes by knock-down. Knipple *et al*., 1994 and Williamson *et al*., 1996 in their work on flies showed mutation corresponding to a substitution of a leucine by a phenylalanine (Leu to Phe) resulting from a single nucleotide polymorphism. This mutation corresponds to a resistance of kdr-type. Results Knipple *et al*. (1994) and Williamson *et al.* (1996) are similar to those of the study. They once again confirm those of this study

CONCLUSION

Study of the larvae strains of *Culex quinquefasciatus* survived to the treatment to Suneem 1% after five generations revealed a resistance due to a mutation of kdr gene. The mutation due to a remplacement of leucin (TTA) to phenylalanine (TTT). The mutation caused overexpression protein of sodium channel gene that is the main site of biopesticides.

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REFERENCES

Abeni ZH, Brown AWA (1960). Cross resistance characteristics of a Malathion tolerant strain developed in Aedes aegypti. Mosq. News, 23 (4): 341-345.

Hamon J, Garret-Jones C (1963). Insecticide Resistance in major malaria vectors and operational importance. Bull. Org. Mond. health (28):1-24.

Haubruge E, Amichot M (1998). The mechanisms responsible for insecticide resistance in insects and mites. Biotechnol. Agron. Soc. Environ. 2(3):161-174.

Hougard JM, Duchon S, Zaim M, Guillet P (1983). Bifenthrin: a useful pyrethroid insecticide for treatment of mosquito nets. J. Med. Entomol. 39:526-533.

Jean-Marc Hougard, Frédéric Darriet, Bakayoko S (1983). Natural environment evaluation of larvicidal activity of Bacillus thuringiensis Say, 1823 and Anopheles gambiae Giles, 1902 sl (Diptera: Culicidae) in West Africa. Cah . ORSTOM , Ser . Ent. med. and Parasitol., 21(2):111-117.

Knipple DC, Doyle KE, Marsella-Herrick PA, Soderlund DM (1994). Tight genetic linkage between the kdr insecticide resistance trait and a voltage-sensitive sodium channel gene in the house fly, Proc. Natl. Acad. Sci. USA 91; 2483-2487.

Lewallen LL, Brown AWA, Gilles PA (1963). Organophosphorus resistance in *Aedes nigromaculis* in California. Mosq. News 23(4):341-345.

Martine-Torres D, Chevillon C, Brun-Barale A, Berge JB, Pasteur N, Pauron D (1999). Voltage dependent Na+ channels in pyrethroid resistant Culex pipiens L mosquitoes. Pesticide Science 55:1012-1020.

Mouchet J, Elliot R, Gariou J, Voelckel J, Varrieras J (1960). Insecticide resistance in Culex pipiens fatigans Wied and hygiene problems in Cameroon. Trop. Med.,. 20(4):448-455.

Narahashi T (1996). Neuronal ion channels as the target sites of insecticides; Pharmacol. Toxicol. 78:1-14.

Ndione RD, Faye O, Ndiaye M, Dieye A, Afoutou JM (2007). Toxic effects of neem products (Azadirachta indica, A. Juss) on Aedes aegypti Linnaeus 1762 larvae. J. Afr. Biotechnol., 6(24):2846-2854.

Ndione RD, Seye F, Ndiaye M, Sow I (2006). Histopathological effects of the products of neem (Azadirachta indica A. JUSS) on larvae of Culex quinquefasciatus Say, 1823 (Diptera: Culicidae). Dakar Médical 51(2):107-112.

Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas I, Bisset J, Rodriguez M, Mccall PJ, Donnelly MJ, Ranson H, Hemingway J, William C. Black IV (2007). A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti. Insect Molecular Biology 16(6):785-798.

Sattelle DB, Yamamoto D (1998). Molecular targets of pyrethroid insecticides. Adv. Insect. Physiol., 20:147-213.

Seye F, Ndione RD, Ndiaye M (2006a). Larvicidal effects of neem products (neem oil and pure Neemix) compared to two chemical synthetic insecticides (Deltamethrin and Fenitrothion) on larvae of Culex quinquefasciatus mosquitoes (Diptera: Culicidae). J. Sci. Technol., 4(1):27-36.

Seye F, Ndione RD, Ndiaye M (2006b). Comparative study of two neem products (oil and powder) on pre-imaginal stages of the mosquito Culex quinquefasciatus (Diptera: Culicidae) . Afr. Sci., 02(2):212-225.

Sinegre G, Jullien JL, Crespo O (1976). Resistance of some populations of Culex pipens (L) Chlorpyrifos (DursbanR) in Languedoc-Roussillon (France). Cahier ORSTOM, Ser. Ent. Med. Parasitol., 14(1):49-59.

Singh OP, Dykes CL, Lather M, Agranat OP, Adak T (2011). Knowdown resistance (kdr)- like mutations in the voltage-gated sodium channel of a malaria vector *Anopheles stephensi* and PCR essays for their detection. Malaria Journal 10:5-9.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol., 30:2725-2729.

Williamson MS, Martinez-Torrez D, Dick CA, Devonshire AL (1996). Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid inscecticides. Mol. Gen. Genet., 252(1-2):51-60