African Journal of Biotechnology Vol. 6 (24), pp. 2846-2854, 17 December, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

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

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Accepted 29 October, 2007

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.

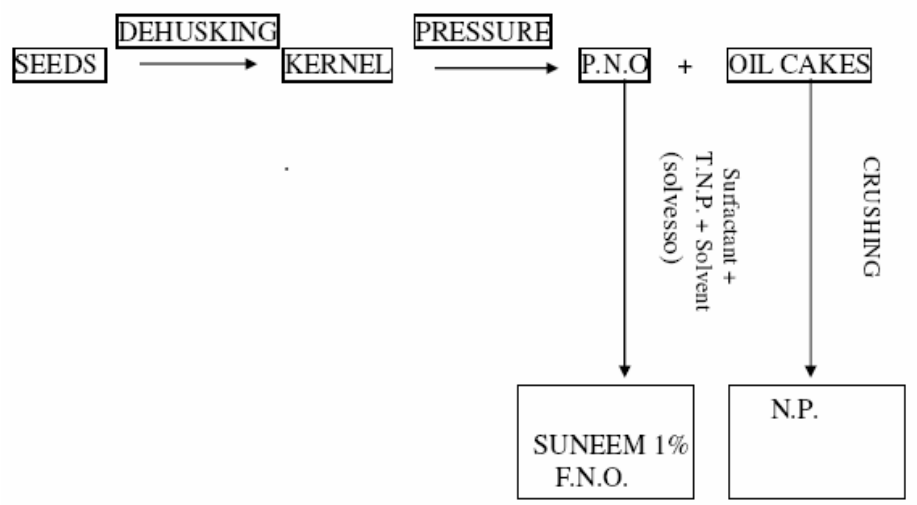

Figure 1. Process to obtain the neem products (***Azadirachta indica***, A. Juss***)*** (Provided by the SENCHIM chemistry's industry Dakar- SENEGAL). P.N.O., pure neem oil; F.N.O., formulated neem oil; T.N.P., technical neem powder; and N.P., neem powder.

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^{o}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).

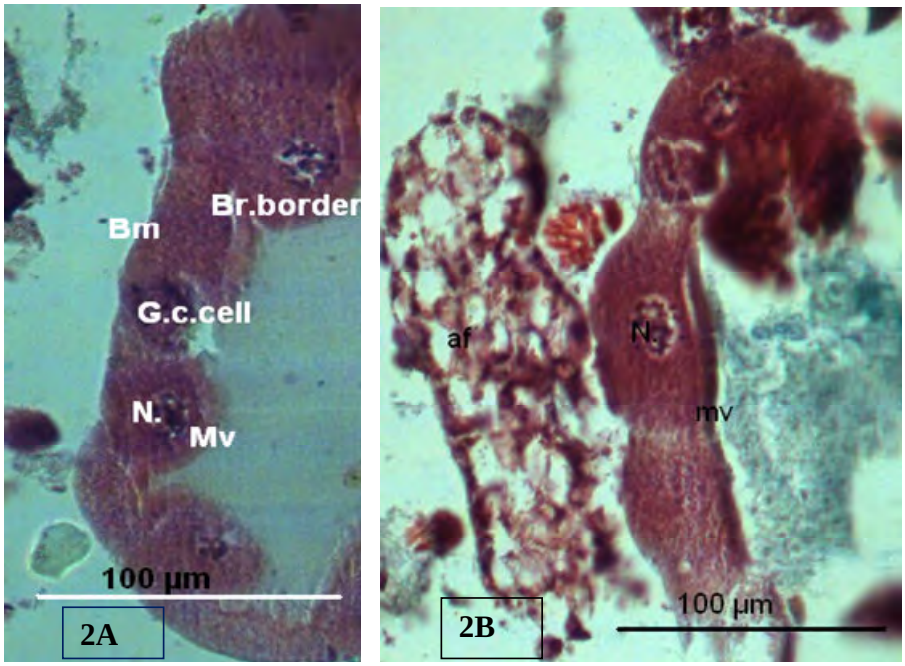

Figure 2. Longitudinal section part in the midgut (Figure 2b) and in the gastric caecum (Ccg). (Figure 2A) of control larva of ***A. aegypti*** Linneaus 1762. One observes nomals epithelias cells with a nucleus (N) that not affected. These cells were laid on a basal membrane (Bm). Adipose fabric (af)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

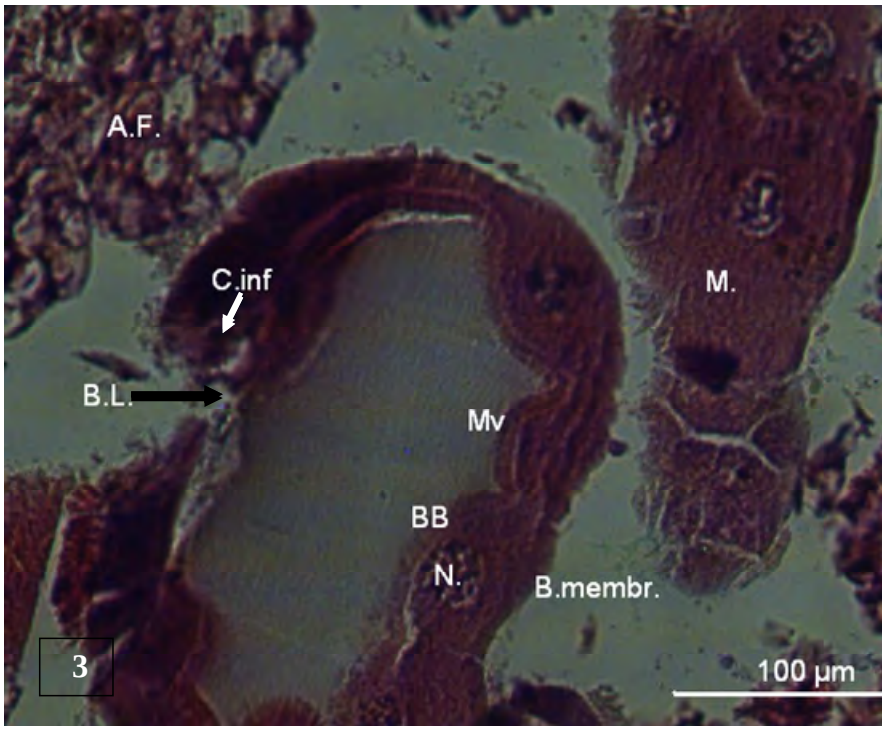

Figure 3. Longitudinal section part of gastric caecum of ***A. aegypti*** Linneaus 1762. This section of larvae treated with neem products showed a morphological lesions of gastric epithelial cells (GEC) with beach lysis (BL). Nuclei (N), muscles (M), adipose fabric (AF) are not affected and seem normal.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

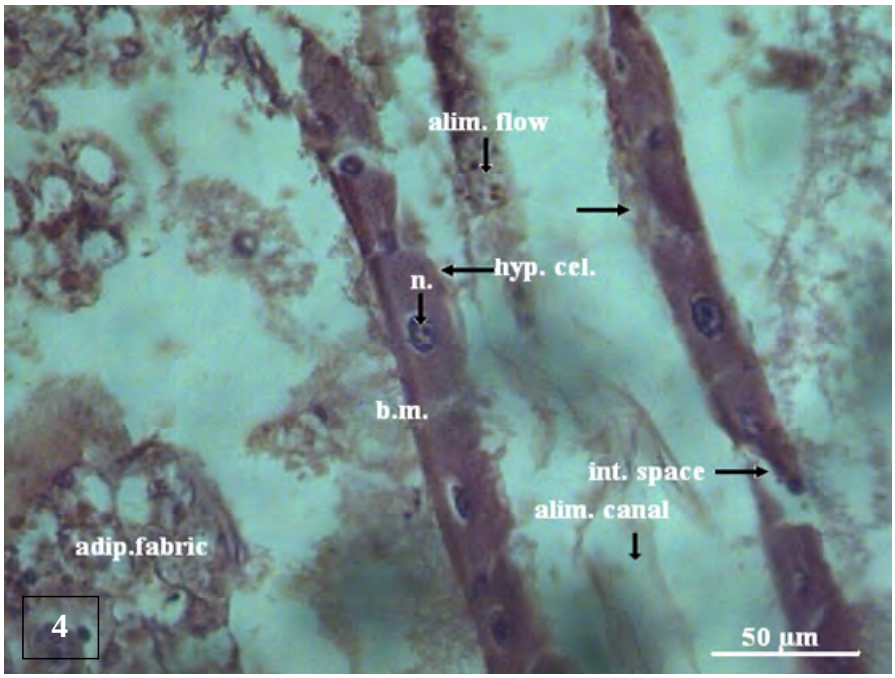

Figure 4. Longitudinal section part of the midgut of 4th instars larvae of ***A. aegypti***. The midgut cells are slightly hypertrophied (arrow; hyp. cel.). Sometimes, we noted an enlargment of intercellular spaces (int. space) and cells lysis. The evident sign consisted here to an absence of alimentary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

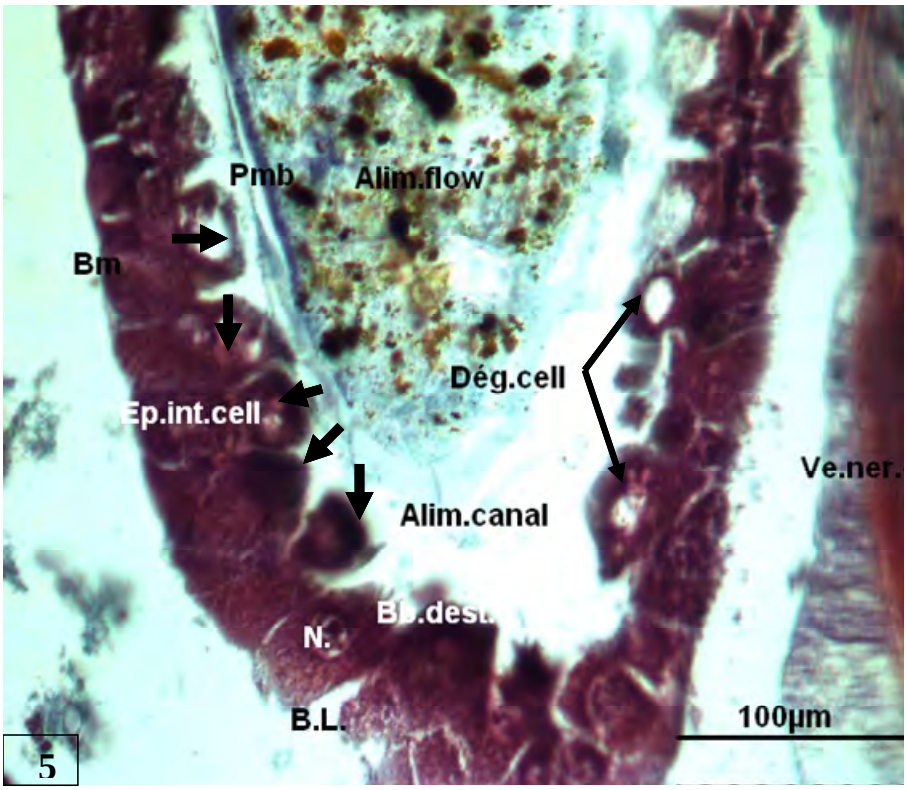

Figure 5. Longitudinal section in posterior part of the gut of ***A. aegypti*** Linneaus 1762 showed that some cells appeared to be degenerated (Dég. cell and arrows). Epithelial intestinal cells (Ep.int.cell) showed a beach of lysis (BL). Cells are in an advanced stage of infection (head of arrows). 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.

ACKNOWLEDGMENTS

We would like to thank the authority of Senchim Industry particularly the manufacturer, Dr Abdoulaye Dieye and the personal of their laboratory for giving us the neem products. We thank D. Ngom and D. Diouf, for their technical help, and my laboratory colleagues F. Seye, M. Toure and G. Diouf for their help in the redaction and correction of this paper.

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