Labaratory Study For The Effect of Neem Oil (*Azadirachta indica*) Against Mosquito Larvae of (*Culiseta longiareolata*) in Wasit Governorate/Iraq.

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دراسة مختبرية لتأثير زيت النيم *Azadirachta indica* ضد يرقات بعوض النوع *Culiseta longiareolata* في محافظة واسط / العراق .

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الخلاصة:

نفذت الدراسة لمعرفة تأثير زيت النيم المستخلص من شجرة *Azadirachta indica* ضد يرقات البعوض من نوع *Culiseta longiareolata* (Macquarrt)وفي جميع المراحل اليرقية, تعرضت اليرقات الى التراكيز (0.001% ,0.01% ,0.1% ,0.5% و 1%) لمدة 24 ساعة و48 ساعة تحت الظروف المختبرية . أثبتت النتائج المختبرية ان زيت النيم كان أكثر سمية تجاه يرقات الطور الاول ووصلت نسبة القتل الى 90% عند التركيز 1% ولمدة 48 ساعة ,بينما في بقية الاطوار اليرقية(الثاني , الثالث والرابع) كانت (86.66% , 46.66% و 43.33%), على التوالي.النتائج الاحصائية لقيم LC50% وضحت بان يرقات الاطوار (الاول , الثاني , الثالث والرابع) كانت (0.713% , 0.748% , 1.588%و 1.797%), على التوالي, بينما قيم LC90% ولنفس الاطوار اليرقية كانت (1.120% ,1.169% ,7.599% و9.516%), على التوالي وبحدود ثقة (95%) خلال 24 ساعة ,وان التركيز القاتل قد أزداد مع الطور اليرقي وفي نفس الفترة فان التركيز LC50% ليرقات الطور الرابع كان يعادل 2.52 مرة من التركيز LC50% ليرقات الطور الاول , كذلك فأن التركيزLC90% ليرقات الطور الرابع يعادل 8.49 مرة التركيز LC90% ليرقات الطور الاول.. في فترة 48 ساعة فان التركيز LC50% ليرقات الطور الرابع كان يعادل 2.61 مرة من التركيز LC50% ليرقات الطور الاول , كذلك فأن التركيزLC90% ليرقات الطور الرابع يعادل 7.53 مرة التركيز LC90% ليرقات الطور الاول. أما نتائج التحليل الاحصائي (كرتل) فقد بينت عن وجود علاقة معنوية موجبة وبكلا المستويين (0.05 و0.01) من المعنوية بين نسبة القتل للاطوار اليرقية وبين التراكيز المستخدمة, وكانت قمتها في الطور اليرقي الاول عندما قيمة الارتباط كانت مساوية الى 0.0001 .

Abstract:

The study was undertaken to investigate the effect of neem oil that extracted from *Azadirachta indica* tree against the larvae of *Culiseta longiareolata* (Macquarrt) mosquito in all larval instars.The larvae were exposed to different concentrations of neem oil (0.001%,0.01%,0.1%,0.5% and 1%) for 24h and 48h under laboratory conditions.Neem oil was more toxic against 1st.larval instar and reached 90%

mortality at concentration of 1% for 48h while the responds of 2^{nd}.,3^{rd}. and 4^{th}. Larval instars were(86.66%,46.66% and 43.33%), respectively.

The statistical results of LC50% value have been shown of the larvae: 1^{st}.,2^{nd}.,3^{rd}. and 4^{th}.instars were (0.713%, 0.748%, 1.588% and 1.797%), respectively. While the LC90% were (1.120%,1.169%,7.599% and 9.516%) , respectively with confidance limits (95%) within 24h . The lethal concentration was increased with larval stage. In the same period the LC50% of the $4^{th.}$ larval instar is 2.52 times the LC50% of the 1^{st}.larval instar, also the LC 90% is equivalent 8.49 times the LC90% of the 1^{st}.larval instar. In 48 h, the LC50% of the $4^{th.}$ larval instar is 2.61 times the LC50% of the 1^{st}.larval instar, also the LC 90% is equivalent 7.53 times the LC90% of the 1^{st}.larval instar.

The results of the statistical analysis(Gretl) showed a significant positive correlation with both levels (0.05 and 0.01) from significant between the mortality ratios of the larval stages and the concentrations used ,the peak was in the larvae of the first instar when the P-value = 0.0001.

Introduction

The primitive use of has led researchers to try to extract and diagnose the active substances found in some plants, which have the effect of extruding or killing insects [1]. Plant species with highly promising insecticidal characteristics belong to the Meliaceae family [2].Neem plant belongs to the genus Azadirachta of which Azadirachta indica (Family:Meliaceae) and its derived products have shown insecticidal property[3]. Neem seeds contain approximately 99 biologically active compounds of which azadirachtin, nimbin, nimbidin and nimbolides are major molecules, Many of these derived products have antifeedancy, ovicidal activity, fecundity suppression besides insect growth regulation and repellency against insects [4]. Mosquitoes are the prime vectors responsible for transmission of diseases to more than seventy billion people annually world wide. As per the reports of World Health Organization, Mosquitoes transmit malaria and alone kills 30 million people annually, the arbo-viruses which cause yellow fever, dengue hemorrhagic fever, epidemic polyarthritis, and several forms of encephalitis eg. Bancroftian filariasis which is caused by a nematode transmitted by mosquito bite [5].

Several studies have examined the effect of Neem extract on larvae of mosquitoes. The neem oil formulation containing 32% neem seed oil (an equivalent of 0.03% azadirachtin), an emulsifier 5% and 63% iso propanol (solvent) was investigated for its larvicidal activities against *Anpheles Gambiae*, and a neem oil had an LC50 value of 11 ppm after 8 days, which was nearly five times more toxic than the corn oil

formulation [6].The neem products also had significant larvicidal activity against *Anopheles gambiae* and the larval mortality was dose dependant [7]. On the other hand, Median lethal concentration (LC_{50}) of neem oil against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* was found to be 1.6, 1.8 and 1.7 ppm ,respectively [8]. The seed oil and leaf extract of neem contain properties that could be developed and used in the control of mosquitoes in the tropics [9]. The *Azadirachta indica* and *Pongamia pinnata* have mosquito-repellent potential [10]. The azadirachtin is promising as a larvicidal agent against larvae of *Culex pipiens* and *Culiseta longiareolata* mosquitoes and could be an alternative of chemical insecticides [11]. Leaves extract of *A .indica* and *D. melei* can be suggested as a natural larvicidal for controlling mosquitoes in India [12]. The main objective of this study is to investigate the toxic effect of neem oil against larvae of *Culiseta longiareolata* mosquitoes of different larval stages to determine the extent to which the larval stages of mosquitoes respond to this plant extract as an alternative to the use of chemical control.

2-Materials and Methods

2-1.Mosquito species Identification .

Culiseta longiareolata (Macquarrt) larvae were collected by standared metallic dipper (USM),which capacity 350 ml from pools that located in the wheat farms near City of Al-Kut. Fourth and most third instar of mosquito were identified to species using identification key [13].As well as the identified results sent to Animal Ecology Labaratory of Science College.Dep. of Biology of Wassit University by Prof.Dr. Jameel AL-Sariy.

2-2.Mosquito rearing

Mosquito larvae were developed in laboratory condition presented with (27-30 C^0) , releative humidity with (70-50%) and photo period (14 hourse/day).Mosquito larvae transferd to Plastic containers500 ml contain on 300 ml chlorinated tap water and 0.03 g of rat food [14]. The water was changed every three days to prevent the formation of the mold on the surface of the water access to the stage of the pupa, which was isolating the state of transformation, and transferred to the plastic containers on water only without food, and placed in a wooden cage cube shape length of 40 cm and coated with tulle to be used in this research for the purpose of conducting larval response experiments [15].

When insects were complete fed with a 10% sugar solution on a cotton saturated with solution in a petri dish inside the breeding cages, and for the purpose of obtaining eggs, the female mosquitoes were fed on the blood of the feather-free pigeon in the chest area [16]. The feeding continues in the dark for 24 hours, the process of

feeding the female on the blood three days after turning into the adult stage [17]. The females eggs boats were collected inside the breeding cages with a small brush and transferred to 500 mL plastic containers on tap water until hatching[18]. For the purpose of obtaining all larval stages for testing with the concentrations used.

2-3 Bioassays

Neem oil was obtained by the Agricultural Research Department / Integrated Control Program Center of the Ministry of Science and Technology. This substance was produced by Padmavati Chemicals.Using 0.2 ml of Tween solution 20%. The following concentrations were prepared(1%, 0.5% , 0.1%, 0.01%,0.001) based on the method [19]. Three replicates were prepared for each concentration in 125 ml of cups containing 100 mL of chlorine-free tap water, 10 larvae were placed in each repeater and the mice diet was fed at 1 ml. The control was in three replicates containing larvae, Tween solution and without adding Neem oil and left for 24-48 hours to know the response of larvae .

2-4 (Statistical analysis)

The percentage mortality were calculated using the formula 1 and corrected mortality using Abbot's(20), when necessary, were done. Lethal concentration for 50% and 90% of the mortality(LC50 and LC90) at 24h untile 48h, with confidence intervals were determined by the probit analysis method as described by Finney [21]. The relationship between the mortality rate of *Culiseta longiareolata* mosquitoe larvae and the concentrations used using Gretl [22] to extract the correlation value (p-value).

Formula **1**

percentage mortality=Number of dead larvae / Number of larvae introduced x 100

3-Results and Discussion

3-1.Effect of Neem oil on 1st. instar larvae of *Culiseta longiareolata* mosquitoes.

The results of the labaratory study on the effect of Neem oil on the first instar larvae of *Culiseta longiareolata* mosquitoes showed high toxicity to the larvae of these stages in period 24h .The mortality rates were based on concentration and time of exposure. The highest mortality rate of these larvae was(83%,16.66%)at 1% and 0.5% concentrations,respectively in period 24h . The highest rate of mortality of these larvae in 48h was (90%, 33.33%,6.66%)at (1%, 0.5% ,0.1%)concentrations,respectively. This may be because the emulsifier allowed the active ingredient in the oil to disperse in the water more easily and evenly, causing the larvae to be exposed to higher concentrations of this substance [23]. The percentage of mortality in this study reached the maximum rate of 90%, especially in the larvae of the first stage at 1%

concentration, as in Fig. (1) . The seeds of the neem plant contain many active substances, the most important of which are azadirachtin and other compounds that are highly concentrated in the early stages such as growth regulators [4]. A role similar to the work of insect hormones for the process of metamorphosis such as juvenile hormones and the moulting hormones maintain the larvae qualities of the insect and prevent the ecdysis of larvae growth[24].The azadirachtin could act as anti-ecdysteroid and kills larvae by growth inhibition effect [25].

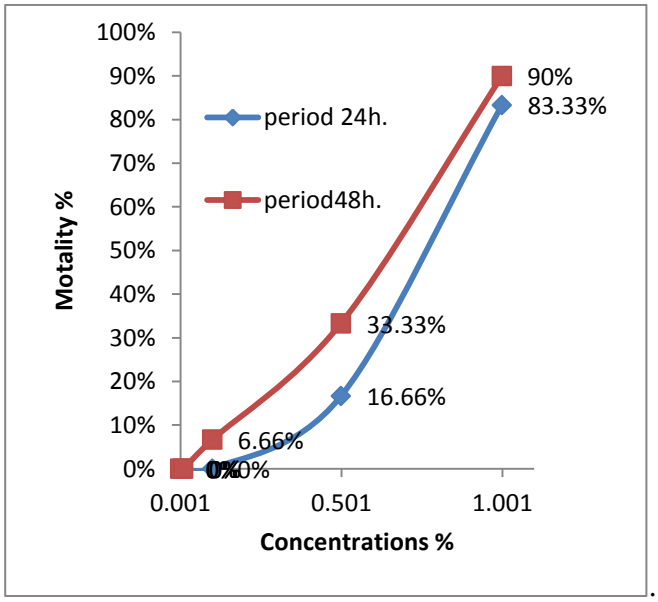
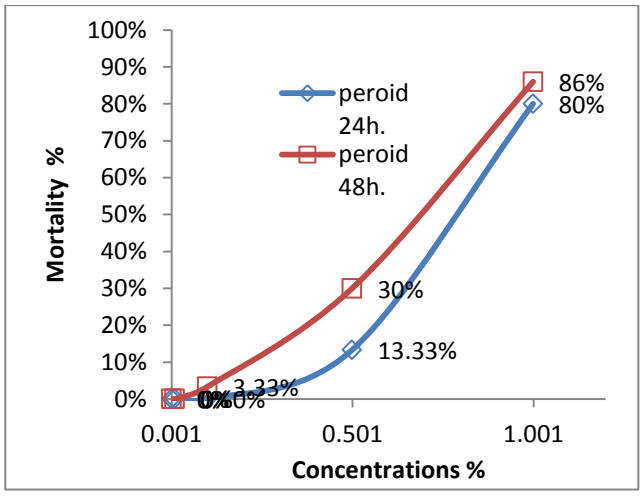


Fig.(1).Dose-response relationship for treatment of neem oil applied for 24h and 48h to 1st. instar larvae of **(*Culiseta longiareolata*)**.

The results of the statistical analysis showed that the lethal concentration of half of the larvae LC50% was 0.713 %, with high confidence limits (95%). The LC90% value is 1.120 % with the same significant for 24h. As for 48 hours , LC50% was (0.608%),while LC90% was(1.008 %) with high confidence limits (95%). The larvae did not respond to other concentrations. As in Table (1).

3-2.Effect of Neem oil on 2nd. instar larvae of *Culiseta longiareolata* mosquitoes.

The results showed that the larvae of the second phase had a response to the Neem oil where the highest rate of mortality was in 24 hours(13.33% ,80%) at 1% and 0.5% concentrations, respectively. The highest rate of mortality of these larvae in 48 hours was (86.66% , 30%,3.33%)at(1% , 0.5% ,0.1%) concentrations,respectively. As in figure (2) .



. Fig.(2).Dose-response relationship for treatment of neem oil applied for 24h and 48h to 2nd.instar larvae of **(*Culiseta longiareolata*)** mosquitoes.

In the 48hours period, the mortality rate was (86.66% , 30% , 3.33%)for concentrations (1%, 0.5%, 0.1%), respectively.The lethal concentration LC50% was 0.628% and for LC90% was 1.077% with confidance limits (95%).The larvae did not respond to the rest of the other concentrations as shown in Table (2).

There is some similarity between the response of the larvae of the first instar and second instar towards specific concentrations of the neem oil extract, especially within 48 hours. It is clear from the foregoing that by increasing the duration of exposure to neem oil, the percentage of mortality increases. The ratio of mortality increases with the cumulative effect when treating domestic flies with the extract of the Tropical Equestrian Plant [26] .

The water extract of the neem seeds with different concentrations gave a mortality ratio of 100% from *Culex's quinquefasciatus* mosquitoes also extended the larval stage in 1st. and 2nd larval instars for this mosquito[27].

(3-3).Effect of Neem oil on 3rd. Instar larvae of *Culiseta longiareolata* mosquitoes.

The results of the study showed that there was a difference in decrease the mortality rates in third larval instar than the mortality rates in the first and second instar, where the response was less. The mortality rates for the larvae of the third instar was (3.33%, 6.66% ,43.33%) for concentrations(0. 1%, 0.5%, 1%) respectively,for 24 hourse.While in the 48h period, the mortality rate was(3.33% , 10% , 46.66%)for concentrations (0.1%, 0.5%, 1%), respectively. As in figure (3).

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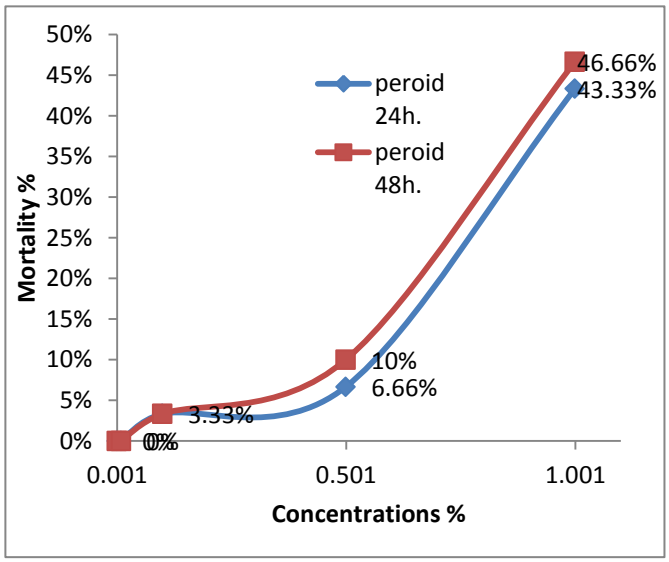


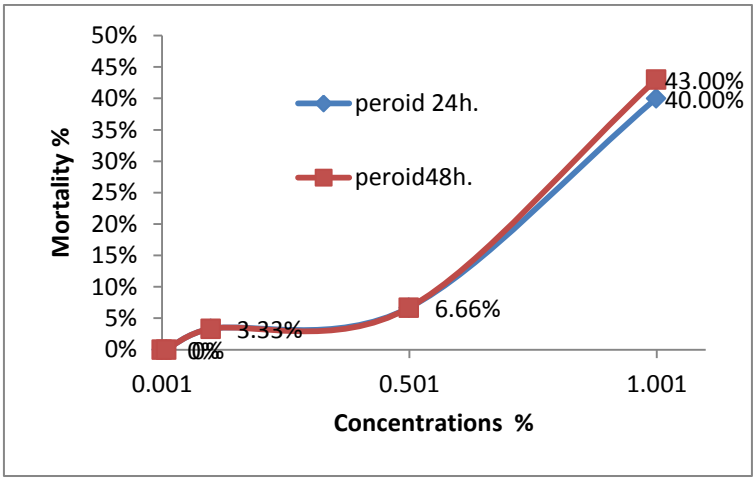
Fig.(3).Dose-response relationship for treatment of neem oil applied for 24h and 48h to 3rd. Instar larvae of **(*Culiseta longiareolata*)** mosquitoes.

The results of the statistical analysis showed that the values of LC50% and LC90% were (1.097%,2.029 %) , respectively in 24h. Both values in 48 hours were (1.380 %,6.330 %) respectively. Other study showed that the Neem oil extract had an effect on the larvae of the third and fourth stages and LC50% was equal to 10.68 ppm for mosquitoes of *Anopheles gambiae* [5].

There is a difference between the results of the present study and the results of some studies, notably because of differences in the origin of products, concentrations of active ingredients of the products, the species of mosquitoes tested, modes of application of the products, and parts of the neem plant from which the products were extracted [6].The larvae did not respond to the rest of the other concentrations as shown in Table (3).

(3-4) Effect of Neem oil on 4th. instar larvae of *Culiseta longiareolata* mosquitoes.

The results of the study revealed that the response of the fourth instar larvae was largely identical with the third instar larvae, especially in the concentrations (0 . 1% , 0. 5%) where the mortality rate was (33% ,6% 66%), respectively . While the concentration of 1% was 40% in 24 hours. The rate of mortality in 48 hours was the same as in the previous in 24hours, except for the concentration of 1% as it was 43 33%. As in Figure (4).



Figure(4).Dose-response relationship for treatment of neem oil applied for 24h and 48h to 4th. Instar larvae of **(*Culiseta longiareolata*)** mosquitoes.

Also the values of of LC50% and LC90% were (1.797%,9.516 %) respectively, in 24h. Both values in 48 hours were (1.588 %,7.599 %), respectively. The larvae did not respond to the rest of the other concentrations as shown in Table (4).

Table (1). The statistical analysis results of response of 1st. instar larvae toward neem oil at different concentrations applied for 24 and 48 hourse.

Concentr-ation %	Mort. % in 24h.	Mort. % in 48h.	LC50 %	**95% confidance limits Lower Upper**	LC90 %	95%confidance limits Lower Upper
Control	0	0				
0.001	0	0				
0.01	0	0				
0.1	0	6.66	0.713	0.612 0.826 in24h.	1.120	0.942 1.570 in24h.
0.5	16.6	33.33				
1	83.33	90	0.608	0.499 0.712 in48h	1.008	0.835 1.512 in 48h.

Table (2). The statistical analysis results of response of 2nd. instar larvae toward neem oil at different concentrations applied for 24 and 48 hourse.

Concent-ration %	Mort. % in 24h.	Mort. % in 48h.	**LC50 %**	**95% confidance limits Lower Upper**	LC90 %	95%confidance limits Lower Upper
Control	0	0				
0.001	0	0				
0.01	0	0				
0.1	0	3.33	0.748	0.644 0.868 in24h.	1.169	0.982 1.647 in24h.
0.5	13.33	30				
1	80	86.66	0.628	0.518 0.738 in48h	1.077	0.883 1.676 in 48h.

Table (3). The statistical analysis results of response of 3rd. instar larvae toward neem oil at different concentrations applied for 24 and 48 hourse.

Concentration %	Mort. % in 24h.	Mort. % in 48h.	LC50 %	**95% confidance limits Lower Upper**	LC90 %	95%confidance limits Lower Upper
Control	0	0				
0.001	0	0				
0.01	0	0				
0.1	3.33	3.33	1.588	0.971 10.824 in24h.	7.599	2.696 1053.288 in24h.
0.5	6.66	10				
1	43.33	46.66	1.380	0.8879 5.686 in48h	6.330	2.488 287.103 in 48h.

Table (4). The statistical analysis results of response of 4th. instar larvae toward neem oil at different concentrations applied for 24 and 48 hourse.

Concentration %	Mort. % in 24h.	Mort. % in 48h.	LC50 %	**95% confidance limits Lower Upper**	LC90 %	95%confidance limits Lower Upper
Control	0	0				
0.001	0	0				
0.01	0	0				
0.1	3.33	3.33	1.797	1.033 20.185 in24h.	9.516	2.998 3850.177 in24h.
0.5	6.66	6.66				
1	40	43.33	1.588	0.971 10.824 in48h	7.599	2.696 1053.288 in 48h.

While [28] that found the Thai. neem oil formulation resulted in 100% mortality among the early fourth stage of *Aedes aegypti* larvae at 48 hours.

The results of the statistical analysis in tables (5 and 6) of the concentration values required for LC50% and LC90% of the *Culiseta longiareolata* mosquito larvae towards neem oil extract showed a positive correlation between the concentration and the larval stage. The lethal concentration of LC50% increased with larval stage, in the same period the LC50% of the 4th. larval instar is 2.52 times the LC50% of the 1st.larval instar, also the LC 90% is equivalent 8.49 times the LC90% of the 1st.larval instar .

In 48 h the LC50% of the fourth stage is 2.61 times the LC50% of the first stage larvae, also the LC 90% for this stage is equivalent 7.53 times the LC90% of the 1st.larval instar , which is consistent with what was found [29]. That indicated a positive relationship between the concentration of nicotin sulfate and the pupal stages of an insect(*Ommatissus binotatus*) which infect palms and it was laboratory .Results of current study show that the first larval stage is more sensitive than the rest of the larvae stages to the neem oil. The reason for the high degree of resistance of the larval stages of the pesticides with age is due to the increase in the thickness of the cuticle[30].

The results of the statistical analysis(Gretl) showed a significant positive correlation with both levels (0.05 and 0.01) from significant between the mortality ratios of the larval stages and the concentrations used. The peak was in the larvae of the first stage when the P-value = 0.0001. as shown in table (7).

Table (5). the concentrations values required for LC50% and LC90% of the *Culiseta longiareolata* mosquito larvae towards Neem oil extract in 24h.

LC90%	LC50%	Larvae instar
1.120	0.713	1st..
1.169	0.748	2nd.
7.599	1.588	3rd.
9.516	1.797	4th.

Table (6). the concentrations values required for LC50% and LC90% of the *Culiseta longiareolata* mosquito larvae towards Neem oil extract in 48h.

LC90%	LC50%	Larvae instar
1.008	0.608	1st..
1.077	0.628	2nd.
6.330	1.380	3rd.
7.599	1.588	4th.

Table (7). The results of the statistical analysis(Gretl) to illustrate the relationship between the mortality rate of *Culiseta longiareolata* mosquitoe larvae and the concentrations used to extract the correlation value.

Larval instars	P-value ***
1st.	0.0001
2nd.	0.0002
3rd.	0.0008
4th.	0.0013

Conclusion:

The present study examines the influence of neem oil that contain (azadirachtin and other compounds) on the toxicity of mosquito larvae of (*Culiseta longiareolata*) in 1st.,2nd.,3rd. and 4th. Larval instars .It has shown that these larval instars had responded to high concentrations only, but the 1st. larval instar was more sensitive to these concentrations. This study showed the ability of neem oil in managing the larvae and

thus contributes as an affordable way to control. Further study is needed for testing this extract in field,and they also need to be tested against other species of mosquitoes, as a alternate for chemical control.

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