Operational use of neem oil as an alternative anopheline larvicide. Part B: environmental impact and toxicological potential

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الاستخدام الميداني لزيت النيم كبديل لمبيدات يرقات البعوض. الجزء الثاني: التأثير على البيئة والتأثير السمي المحتمل

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الخلاصة: أجريت الدراسة للاستكشاف المبدئي لسمية زيت نيم، وزيت التيمفوس، وميثيل كلور بيريفوس والفينتروثيون على الثدييات وعلى البيئة. وقد استخدمت أنواع البعوض الأنبوبية، والدافنية الكبيرة والغمبوزية النهمة لدراسة تأثير مبيدات البعوض على البيئة. وقد لوحظ مستوى مرتفع من السمية مع اختلاف طفيف بين الكائنات الحية؛ فبعد معالجة تستمر لمدة تسعين يوماً بزيت النيم الخام بمقدار 5 ميلي غرام لكل كيلو غرام من وزن البدن لفئران المختبرات لم تتسبب بأي تغييرات ذات أهمية في وزن الجسم كل أسبوع، وفي مؤشرات تلف الكبد في السيروم (المصل) ولا في مستوى البيلروبين المباشر والبيلروين الكلي. و لم تكن قيم متثابتات الدم في الفئران المعالجة مختلفة طيلة تسعين يوماً عما لدى فئران المراقبة. وبالمجمل فإن استخدام زيت النيم الذي يعد مصادقاً للبيئة كبديل لمبيدات يرقات البعوض يبدو أمراً مقبولاً.

ABSTRACT This study was conducted to investigate the preliminary environmental and mammalian toxicology of neem oil, temephos and chlorpyriphos-methyl/fenitrothion. *Culex pipiens*, *Daphnia magna* and *Gambusia affinis* were used to study environmental impact. A high level of toxicity was observed, with slight differences between organisms. The emulsifiers individually also displayed toxicity towards the tested organisms. Up to 90 days daily oral crude neem oil treatment (5 g/kg body weight) of laboratory mice did not cause any significant changes in weekly body weight gain, nor in serum liver damage indicators, direct bilirubin or total bilirubin. Blood parameters of treated mice up to 90 days were not statistically different from those of control mice. Neem oil could be used as an environmentally friendly alternative to the traditional chemical anopheline larvicides.

Utilisation opérationnelle d'huile de margousier comme autre larvicide d'anophèle. Partie B : impact sur l'environnement et potentiel toxicologique

RESUME Cette étude a été réalisée pour examiner la toxicologie préliminaire de l'huile de margousier, du téméphos, et du chlorpyrifos-méthyl/fénitrothion pour l'environnement et chez les mammifères. *Culex pipiens*, *Daphnia magna* et *Gambusia affinis* ont été utilisés pour étudier l'impact sur l'environnement. Une toxicité importante a été observée avec de légères différences entre les organismes. Les émulsifiants présentaient aussi individuellement une toxicité pour les organismes testés. Un traitement d'huile de margousier brute administré quotidiennement par voie orale pendant une période maximale de 90 jours (5 g/kg de poids corporel) à des souris de laboratoire n'a entraîné aucun changement important dans la prise de poids hebdomadaire, ni dans les indicateurs sériques d'atteinte hépatique, la bilirubine directe ou la bilirubine totale. Les paramètres sanguins des souris traitées pendant une période maximale de 90 jours n'étaient pas statistiquement différents de ceux des souris témoins. En conclusion, l'utilisation d'huile de margousier, substance plutôt inoffensive pour l'environnement, est une alternative au larvicide d'anophèle.

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Introduction

Economic and environmental concerns have encouraged a tendency recently towards the use of "soft" pesticides. Products of the neem tree (*Azadirachta indica*) are described as being remarkably benign to most beneficial organisms [*1*]. It has been suggested, however, that, depending on the application rate and environmental fate, pesticides based on azadirachtin (the main active ingredient of neem) may have direct adverse effects on aquatic organisms and that the toxicity and stability of formulated pesticides depend on factors other than azadirachtin concentration alone [*2*]. Although natural insecticides from the neem tree are generally perceived as less harmful to the environment than synthetic insecticides, new evidence indicates that these products may pose a risk to certain non-target organisms [*3*]. NeemAzal-T/S (10g/L azadirachtin) exhibited toxicity to mosquito larvae as well as to certain non-target organisms. The order of tolerance of the organisms to different concentrations of the insecticide was: *Bufo regularis* larvae (Amphibia) > *Aedes caspius* (Insecta) > *Gambusia affinis* (Poeciliidae) > *Cyclops* sp. > *Daphnia magna* (Crustacea). At a concentration of 20 ppm, all the tadpoles died within 9 days, while all other individuals died within 5–8 days after exposure to a concentration of 10 ppm [*4*]. Chronic tests of neem-based pesticides on *Culicidae* larvae, however, showed greater toxicity in the laboratory exposures than *in situ*.

No significant mortality occurred after testing 2 neem-based formulations on 8 species of macro-invertebrates in flow-through screening tests at 10 times the expected environmental concentration (0.35 ppm). At longer exposures of 0.035 ppm in aquatic microcosms, no significant mortality or antifeedant effects were observed after a 28-day exposure [*5*].

It has been found that application of neem-based pesticides at recommended application rates did not harm aquatic invertebrates categorized as planktonic and filter feeding (*Culex* sp. and *Daphnia* sp.). The benthic invertebrate *Chironomus riparius* was, however, affected by multiple applications of neem. This demonstrates that that the use of neem extracts in or near aquatic environments may lead to disturbances in benthic populations, and may cause decreases in numbers of organisms that are important in food web and nutrient cycling processes [*2,3,6,7*].

Azadirachtin, the main pesticidal active component of neem, administered to male and female rats at doses between 500 and 1500 mg/kg per day for 90 days did not produce any signs of toxicity, mortality, changes in tissue weight, pathology or serum and blood parameters [*8*].

Acute oral toxicity of neem oil has, however, been documented in rats and rabbits. Dose-related pharmacotoxic symptoms were noticed along with a number of biochemical and histopathological indices of toxicity. The 24-hour LD_{50} was established as 14 mL/kg in rats and 24 mL/kg in rabbits. Prior to death, animals of both species exhibited pharmacotoxic symptoms comparable in order and severity, with lungs and central nervous system as the target organs of toxicity [*9*]. Vepacide (a neem-based pesticide) was reported to increase alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rat serum, kidney and lung and to decrease these enzymes in the liver when administered for 45 and 90 days [*10*]. Also, neem oil increased blood ALT and AST after 15 hours of oral administration in male rats [*11*]. Aqueous extract of the leaf, however, reduced the elevated serum ALT, AST, and gamma-glutamyltransferase (GGT) in rats with paracetamol damaged livers [*12*]. Biochemical and histological effects of neem

on the reproductive organs of rats and on their reproductive potential have also been demonstrated [*13–15*].

This study investigates the preliminary environmental and toxicological impact of neem extract on non-target species *C. pipiens, D. magna* and *G. affinis* and mammalian toxicology on albino laboratory mice.

Methods

Field *Culex pipiens, Daphnia magna* and *Gambusia affinis*

Field strains of *C. pipiens, D. magna* and *G. affinis* were collected from various locations in El Henawy village and Abheit El Hagar village, Sinnuris district, Fayoum governorate and from various locations in agricultural areas in Alexandria governorate. Susceptibility tests for neem oil (Plasma Power Private Limited, Chennai, India; azadirachtin content 1570 ppm), corn oil, FEBA dish washing detergent (Alexandria Company for Chemicals and Detergents, Alexandria, Egypt) (used as emulsifier), temephos (purchased as Bordin EC for public health purposes [temephos 50%, berol 11%, emulsifiers and white kerosene 39%], Al Helb for Pesticides and Chemicals, New Domiat, Egypt) and chlorpyriphos-methyl/fenitrothion (Chlorosol EC [chlorpyriphos-methyl 20%, fenitrothion 20%, emulsifiers and solvents 60%], Kafr El Zayat Company for Pesticides and Chemicals, Kafr El Zayat, Egypt) were carried out on these strains according to standard methods [*16–18*]. *D. magna* and *G. affinis* had been acclimatized for 1 week in the laboratory before the experiment.

Preliminary toxicological study of neem oil on laboratory mice

Three-month-old male laboratory mice were put in cages, 5 animals to each. Commercial balanced diet and drinking water were provided *ad libitum*. Toxicity of neem oil to laboratory mice was tested at doses ranging from 1 g/kg body weight to 25 g/kg body weight. No mortality was observed until 7 days post treatment.

To study the toxicology in mice, laboratory mice were treated with neem oil at dosage of 5 g/kg body weight/day (mixed with food) up to 90 days. Toxicity was determined for concentrations of emulsifiers that are normally used to emulsify the neem oil or the vegetable oil (20% of the neem oil concentration for FEBA and 5% of the neem oil concentration for Triton X-100). The mice were divided into 5 groups, each consisting of 40 mice, 20 for treatment and another 20 as controls. The groups were treated for 1 day, 1 week, 1 month, 2 months and 3 months. The mice were weighed weekly and body weight gain was calculated. On the day of slaughter animals were weighed then a blood sample was taken from the tails on a glass slide for leukocyte differentiation. Blood samples were also taken for erythrocyte and leukocyte counts. Haematocrit tubes were filled for determination of erythrocyte volume [*19*]. Haemoglobin determination was done using kits (Diamond Diagnostics, Holliston, Massachusetts). A 20 µL sample of heparinized blood was mixed with 5.0 mL of working solution. Absorbance of standard and samples was read against working solution after 5 minutes. Haemoglobin concentration (mmol/L) was calculated according to the formula provided ($A_{sample} \times 22.82$).

About 1 mL of blood was put into a centrifuge tube for centrifugation. Serum was collected and kept below 20 °C for determination of total bilirubin, direct bilirubin, AST, ALT and prothrombin time. Diamond Diagnostics kits were used for bilirubin and enzyme determination and Thromborel S kits for prothrombin time determination (Dade Behring, Marburg, Germany). Al-

though each group consisted of 20 treated animals and 20 controls, only 10 animals from each group were randomly chosen for each parameter determination due to the small amount of blood that could be taken from each mouse.

Results

Impact of larvicides on *C. pipiens*

Neem oil displays a certain level of toxicity against *C. pipiens* larvae. LC_{50} after 24 hours was 91.6 mg/L (95% CI: 63.2–132.3) for the Fayoum strain and 128.2 mg/L (95% CI: 90.9–180.4) for the Alexandria strain. The Alexandria strain tolerated neem oil and vegetable oil better than the Fayoum strain after 24 hours of exposure. In contrast, after 48 hours of exposure, the Fayoum strain was more tolerant to both. The difference in susceptibility of the 2 strains to FEBA dishwashing detergent was clear. The LC_{50} was 68.7 mg/L (95% CI 50/2–94.3 for the Alexandria strain and 171.5 mg/L (95% CI 21.8–1418.5) for the Fayoum strain (Table 1).

The susceptibility of the Alexandria strain to temephos was almost twice that of the Fayoum strain after 24 hours of exposure, LC_{50} 1.4 µg/L (95% CI: 1.2–1.8) and 2.5 µg/L respectively (95% CI: 0.6–12.5), while after 48 hours the trend was reversed. Both strains showed the same response to chlorpyriphos-methyl/fenitrothion (LC_{50} 1.2 µg/L) (Table 1).

Impact of larvicides on *D. magna*

D. magna originating from water bodies in Fayoum and Alexandria governorates showed almost the same response to neem oil, vegetable oil, FEBA detergent and Triton X-100 with a very slight tolerance in the Alexandria strain (Table 2). Susceptibility of *D. magna* to Triton X-100-based neem oil emulsion was greater than that of FEBA detergent-based neem oil emulsion. Toxicity of these substances to *D. magna* was more or less of the same order as to *C. pipiens* (Tables 1 and 2).

Impact of larvicides on *G. affinis*

Toxicity of neem oil to *G. affinis* originating from water bodies in Sinnuris district, Fayoum governorate and from Alexandria governorate showed that the Alexandria strain tolerated neem oil, temephos and chlorpyriphos-methyl/fenitrothion better than the Fayoum strain (Table 3). Toxicity of neem oil, temephos, and chlorpyriphos-methyl/fenitrothion to *C. pipiens*, and *D. magna* was in approximately the same range (Tables 1–3).

Preliminary toxicological study of neem oil on laboratory mice

Daily oral pure neem oil treatment of laboratory mice up to 90 days did not cause any significant changes in weekly body weight gain. Neither did it cause any significant changes in serum ALT, AST, direct bilirubin or total bilirubin, except a significant decrease in serum direct bilirubin after 1 week of treatment and a significant increase in total bilirubin after 2 months of treatment (*t*-test) (Table 4). Moreover blood parameters—haemoglobin content, erythrocyte count, leukocyte count, prothrombin time, haematocrit (packed cell volume), mean corpuscular volume, mean cell haemoglobin, and mean cell haemoglobin concentration—of treated mice up to 90 days did not show any statistical difference compared to those of control mice (*t*-test) (Table 5). Also, no difference was found for white blood cell differential count for treated and control animals (Table 6).

Table 1 **Toxicity of neem oil, temephos, vegetable oil and chlorpyriphos-methyl/fenitrothion with different emulsifiers to *Culex pipiens* larvae originating from water bodies in Fayoum governorate and Alexandria governorate**

Treatment (mg/L)	Time (hours)	*C. pipiens* Fayoum strain Slope	LC_{50}	95% CI	LC_{95}	95% CI	*C. pipiens* Alexandria strain Slope	LC_{50}	95% CI	LC_{95}	95% CI
Neem oil + detergent[a]	24	2.27	91.6	63.2–132.3	486.4	242.9–972.8	1.70	128.2	90.9–180.4	1197.1	482.0–2966.7
	48	2.82	65.4	40.6–104.9	241.8	137.0–463.1	2.11	54.7	32.9–90.1	329.7	208.7–517.9
Vegetable oil + detergent[a]	24	1.75	137.1	94.4–198.9	1199.1	310.7–4665.2	2.06	190.4	148.7–243.6	1204.9	545.3–2652.3
	48	2.03	93.5	60.2–143.6	607.5	246.7–1498.4	1.41	60.2	30.1–119.2	896.9	332.8–2418.6
Detergent only	24	1.93	171.5	21.8–1418.5	1226.8	956.4–1760.9	2.15	68.7	50.2–94.3	402.5	128.9–1265.2
	48	1.16	113.0	22.8–606.7	2945.6	1133.1–10119.7	1.83	28.6	21.1–38.6	226.1	98.9–520.2
Neem oil + Triton X-100[a]	24	ND	ND	–	ND	–	3.56	568.3	478.9–674.3	1648.6	948.0–2853.1
	48	ND	ND	–	ND	–	2.02	303.2	211.7–433.4	1976.9	765.0–5081.6
Vegetable oil + Triton X-100[a]	24	ND	ND	–	ND	–	3.78	701.1	571.9–859.7	1911.3	975.1–3729.6
	48	ND	ND	–	ND	–	2.59	434.0	348.9–539.8	1882.9	904.4–3898.8
Triton X-100 only	24	ND	ND	–	ND	–	7.14	155.9	134.4–180.8	265.2	168.5–416.4
	48	ND	ND	–	ND	–	6.87	123.4	111.6–136.6	214.5	163.1–281.2
Temephos[b]	24	0.94	2.5	0.6–12.5	138.7	0.9–1373.4	4.60	1.4	1.2–1.8	3.3	2.0–8.5
	48	1.48	0.4	0.2–0.6	4.76	1.6–38.0	3.52	0.9	0.3–1.4	2.8	1.1–48.3
Chlorpyriphos-methyl/ fenitrothion[b]	24	1.61	1.2	0.5–5.1	12.7	1.1–1688.0	1.74	1.2	0.1–1.7	11.1	5.8–17.0
	48	1.44	0.5	0.3–0.8	6.5	1.1–123.8	2.45	0.6	0.4–0.9	3.0	0.8–10.2

[a]Detergent (FEBA) constitutes 20% or Triton X-100 constitutes 5% of the oil concentrations used in the emulsion.
[b]µg/L.
ND = not determined.

Table 2 **Toxicity of neem oil with different emulsifiers to *Daphnia magna* originating from water bodies in Fayoum governorate and Alexandria governorate**

Treatment (mg/L)	Time (hours)	*D. magna* Fayoum strain Slope	LC_{50}	95% CI	LC_{95}	95% CI	*D. magna* Alexandria strain Slope	LC_{50}	95% CI	LC_{95}	95% CI
Neem oil + detergent[a]	24	1.59	168.6	114.4–249.8	1836.5	484.9–7040.5	2.57	166.4	117.2–236.8	727.4	242.1–2191.7
	48	1.98	63.9	47.5–85.7	432.2	229.9–811.7	2.12	57.5	43.1–76.6	343.1	188.2–626.2
	72	1.87	36.2	24.9–52.3	275.4	159.6–475.4	ND	ND	–	ND	–
	96	2.04	17.8	9.4–32.7	113.7	51.2–260.2	ND	ND	–	ND	–
Vegetable oil + detergent[a]	24	5.06	182.9	153.9–217.3	387.1	235.1–635.3	4.07	299.6	117.2–769.9	762.5	97.7–5990.7
	48	2.89	126.2	103.7–153.5	469.1	253.2–865.8	2.24	271.1	132.8–558.3	1473.1	215.3–10224.7
	72	2.33	72.9	55.3–95.9	372.3	192.3–720.7	ND	ND	–	ND	–
	96	2.32	30.9	20.4–46.4	159.0	89.6–283.7	ND	ND	–	ND	–
Detergent only	24	5.74	51.6	32.7–81.7	99.9	33.5–298.3	2.95	82.1	16.2–429.6	297.1	9.5–9897.5
	48	2.22	69.6	29.8–165.1	385.7	37.5–4103.5	3.54	52.0	25.9–105.3	152.2	27.6–856.4
	72	2.84	32.9	26.0–41.7	125.4	54.6–289.0	ND	ND	–	ND	–
	96	2.32	24.8	1 9.4–31.8	128.1	53.7–308.2	ND	ND	–	ND	–
Neem oil + Triton X-100[a]	24	1.86	68.0	50.5–92.3	532.2	236.9–1161.9	3.34	72.3	57.8–90.2	225.0	148.7–339.6
	48	1.32	14.0	5.9–32.0	252.0	85.0–782.2	2.85	40.0	27.0–58.9	150.9	96.3–236.1
	72	1.26	3.3	0.3–30.3	66.9	20.4–240.1	3.13	30.6	19.1–48.6	102.7	67.3–156.7
	96	1.32	2.1	6.8–228.9	37.0	6.5–257.1	4.66	27.88	3.7–191.9	62.9	6.7–671.8
Vegetable oil + Triton X-100[a]	24	1.49	161.6	98.6–267.0	2059.8	387.9–11261.3	2.73	160.8	116.9–221.7	645.1	238.4–1747.9
	48	1.24	37.2	22.2–61.7	788.1	223.9–2872.9	4.54	94.7	113.8–78.8	218.4	151.8–313.2
	72	1.21	22.9	11.7–43.6	527.3	166.2–1736.3	3.19	55.0	75.1–40.2	181.1	117.2–279.1
	96	0.95	5.9	1.1–27.1	313.0	82.5–1275.7	3.04	41.4	56.9–25.7	144.5	80.5–259.9

Table 2 **Toxicity of neem oil with different emulsifiers to *Daphnia magna* originating from water bodies in Fayoum governorate and Alexandria governorate** (*concluded*)

Treatment (mg/L)	Time (hours)	Slope	*D. magna* Fayoum strain LC_{50}	95% CI	LC_{95}	95% CI	Slope	*D. magna* Alexandria strain LC_{50}	95% CI	LC_{95}	95% CI
Triton X-100 only	24	3.78	32.1	25.3–40.9	87.6	43.4–177.6	1.22	32.5	80.5–13.8	734.9	13.3–55465.4
	48	2.2	10.3	7.7–13.7	57.7	33.6–100.6	1.93	16.7	25.7–10.8	119.2	28.4–533.2
	72	1.96	6.78	4.5–10.0	46.8	23.6–97.2	2.65	12.9	19.3–8.6	54.1	25.4–117.4
	96	1.96	3.7	2.1–6.0	25.4	13.8–49.7	3.08	12.1	19.6–8.3	41.7	23.3–75.3

[a] *Detergent 20%, Triton X-100 5% of the oil concentration in the emulsion.*
ND = not determined.

Discussion

Impact of neem oil and other traditional larvicides on non-target aquatic organisms

Non-target organisms originating from water bodies in Sinnuris district, Fayoum governorate and cultivated areas in Alexandria governorate showed slightly different responses with regard to toxicity of the tested substances. The LC_{50} of neem oil emulsified with detergent for *C. pipiens* was 91.6 after 24 hours and 65.4 mg/L after 48 hours for the Fayoum strain and 128.2 and 54.7 mg/L for the Alexandria strain, compared with LC_{50} of about 20 mg/L towards anopheline larvae [*20*]. When Triton X-100 was used as an emulsifier, the LC_{50} for the Alexandria strain was 568.4 and 303.2 mg/L (Table 1).

For *D. magna*, the neem oil-FEBA emulsion LC_{50} for 24 hours was 168.648 mg/L for the Fayoum strain and 166.4 mg/L for the Alexandria strain. After 48 hours, these were 63.9 mg/L and 57.5 mg/L respectively. The LC_{50} for neem oil-Triton X-100 emulsion after 24 hours was 68.0 mg/L for the Fayoum strain and 72.3 mg/L for the Alexandria strain. These values were 14.0 mg/L (48 hours) 3.3mg/L (72 hours) and 2.1 mg/L (96 hours) for Fayoum and 40.0 mg/L (48 hours) 30.6 mg/L (72 hours) and 27.9 mg/L (96 hours) (Table 2). Neem oil and corn oil were found to have almost the same toxicity when emulsified with either FEBA or Triton X-100. Moreover, the toxic effect of Triton X-100 was more obvious than that of FEBA.

Neem oil emulsified with FEBA was less toxic to *G. affinis* than chlorpyriphos-methyl/fenitrothion and temephos (Table 3).

Surprisingly, vegetable oil showed a considerable level of toxicity towards the tested organisms as well. It was slightly

Table 3 **Toxicity of neem oil with FEBA detergent, temephos and chlorpyriphos-methyl/fenitrothion to *Gambusia affinis* originating from water bodies in Sinnuris district, Fayoum governorate and in Alexandria governorate**

Treatment	Time (hours)	*G. affinis* Fayoum strain					*G. affinis* Alexandria strain				
		Slope	LC_{50}	95% CI	LC_{95}	95% CI	Slope	LC_{50}	95% CI	LC_{95}	95% CI
Neem oil + detergent[a] (mg/L)	24	6.70	92.2	79.8–106.6	162.5	99.4–265.1	5.17	160.7	134.5–192.1	334.7	223.5–499.9
	48	7.90	81.3	73.3–90.3	131.5	97.6–176.9	3.97	124.1	96.2–159.8	322.8	196.6–529.0
	72	7.30	76.0	68.1–84.9	127.7	96.4–168.9	2.88	115.6	79.8–167.1	431.0	186.2–999.4
	96	9.50	70.6	58.0–86.0	105.3	75.2–147.2	1.94	84.3	44.0–159.9	598.4	138.5–2635.6
Temephos (µg/L)	24	3.53	1.9	1.4–5.3	5.5	2.4–122.9	ND	ND	–	ND	–
	48	3.89	1.2	1.0–1.6	3.2	2.0–18.1	ND	ND	–	ND	–
	72	4.38	1.1	0.9–1.3	2.6	1.8–8.9	ND	ND	–	ND	–
	96	4.18	0.9	0.7–1.1	2.3	1.6–9.6	ND	> 10	–	ND	–
Chlorpyriphos-methyl/fenitrothion (µg/L)	24	19.02	3.2	2.9–3.5	3.9	3.3–4.7	ND	ND	–	ND	–
	48	21.13	3.0	2.7–3.3	3.6	3.0–4.2	ND	ND	–	ND	–
	72	24.20	2.6	1.9–3.5	3.0	1.7–5.5	ND	ND	–	ND	–
	96	ND	> 2.5	–	ND	–	ND	> 12	–	ND	–

[a]*Detergent constitutes 20% of the oil concentration.*
ND = not determined.

Table 4 **Impact of daily neem oil oral treatment (5 g/kg body weight) on mouse serum AST, ALT, direct bilirubin and total bilirubin**

Exposure time	Serum AST (IU/L)		Serum ALT (IU/L)		Serum direct bilirubin (mg/dL)		Serum total bilirubin (mg/dL)	
	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*
1 day	65.00	9.58	18.60	4.31	2.46	1.58	2.89	1.67
Control	62.90	4.95	19.75	1.83	2.56	0.54	2.96	1.20
1 week	64.30	12.07	17.25	5.55	1.60*	1.43	2.56	1.87
Control	63.80	4.26	16.10	1.71	2.73	0.78	2.61	0.87
1 month	62.70	11.66	16.45	6.45	3.17	1.44	2.97	1.39
Control	73.40	16.45	17.40	4.70	2.85	1.77	2.38	1.20
2 months	58.60	14.31	27.25	1.90	2.01	1.48	2.60*	0.52
Control	53.10	8.58	24.60	12.06	2.38	0.65	1.78	0.67
3 months	65.20	21.19	27.10	6.78	3.32	2.64	1.92	1.04
Control	79.30	10.29	32.15	12.12	3.06	1.41	2.79	1.76

s = standard deviation.
AST = aspartate aminotransferase.
AST = alanine aminotransferase.
**t-test significantly different from control group at 5% level of significance.*

lower than that of neem oil under the same conditions. FEBA detergent or Triton X-100 when used individually also showed toxicity towards the tested organisms (Tables 1–3). The concentration of emulsifier used is only 5% of the oil in the case of Triton X-100 and 20% in the case of FEBA. Such results indicate the "non-spe-cificity" of neem oil as a larvicide or as toxicant to non-target organisms, indicating it worked mainly with its physical properties rather than having a systemic action resulting from azadirachtin content.

In 1998 the US Environmental Protection Agency recorded the LC_{50} of dihydroazadirachtin against *D. magna* to be 11.625 mg/L and against rainbow trout and bluegill sunfish to be 17.65 mg/L and 9.0 mg/L respectively [*21*]. Such concentrations are high, and might be due to rapid photodegradation of azadirachtin A and azadirachtin A_2 [*22*,*23*]. However, many authors have demonstrated the toxicity of neem-based industrial formulations to several non-target organisms, including macroinvertebrates such as *D. magna* and *D. pulex* [*1–7*]. Additives included in the formulation could be the cause of such high toxicity towards the non-target organisms. Neutralized alkyl benzene sulfonate, a synthetic detergent, produced toxicological response on aquatic ecosystems. The median tolerance limit at 0, 24, 48, 72 and 96 hours demonstrated that the water flea (*D. magna*) is more susceptible to the detergent than fish fingerlings (*Cirrhina mrigala*), tadpoles (*Rana cyanophlyctis*), slug worms (*Tubifex rivulorum*), snails (*Lymnaea vulgaris*), and mosquito larvae (*C. pipiens*) [*24*].

Table 5 **Impact of daily oral neem oil treatment (5 g/kg body weight) on mouse blood parameters**

Exposure time	Haemoglobin (mmol/L)		Erythrocytes (10^6/mm^3)		Leukocytes (/mm^3)		Haematocrit (%)		Prothrombin time (s)		MCV (μm^3)		MCH (pg)		MCHC (%)	
	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*
1 day	9.92	3.16	10.07	1.46	5872**	1529	64.9*	5.86	97.44	99.68	32.78	5.03	16.17	6.13	49.22	14.86
Control	9.34	1.07	9.76	1.65	9948	988	60.10	2.47	82.46	94.39	32.88	5.87	15.78	2.98	48.07	5.52
1 week	13.17	2.67	9.44	1.16	8855*	1271	59.00	5.69	93.63	91.75	32.14	5.19	22.71	5.28	71.06	13.17
Control	12.79	2.63	9.58	1.19	9923	758	7.00	4.89	82.67	48.85	30.56	3.20	22.02	6.19	71.48	17.48
1 month	15.88	2.37	9.72	1.83	9971	1455	50.70	7.10	23.23	21.98	27.59	9.73	27.62	8.77	102.22	19.97
Control	13.90	2.26	9.64	1.20	9970	1375	56.50	6.13	16.18	2.75	29.74	4.57	23.56	5.05	80.53	17.91
2 months	8.55	2.99	5.43	2.30	8097	2243	32.90	13.62	45.24	52.49	77.74	62.07	32.43	30.34	47.52	20.69
Control	9.79	4.49	6.45	2.78	6849	1812	37.70	11.34	62.27	59.88	72.03	42.42	24.86	6.81	45.21	23.99
3 months	6.23	1.65	7.66*	1.49	11107	2299	64.40	8.68	37.95	31.45	43.64	9.55	13.31	3.37	31.83	10.21
Control	7.67	2.18	9.12	1.31	9932	1269	67.80	9.11	39.78	28.54	37.90	7.93	13.97	5.34	36.48	9.74

MCV = mean corpusclar volume.
MCH = mean cell haemoglobin.
MCHC = mean cell haemoglobin concentration.
s = standard deviation.
**t-test significantly different from control group at 5% level of significance.*
***t-test significantly different from control group at 1% level of significance.*

Table 6 **Impact of daily oral neem oil treatment (5 g/kg body weight) on differential cell count of mouse leukocytes**

Time of exposure	Eosinophils %		Basophils %		Neutrophils %		Monocytes %		Lymphocytes %	
	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*
1 day	5.95	1.38	4.75	1.44	50.70	2.64	9.10	1.08	29.70	4.12
Control	5.80	0.92	5.10	1.20	50.80	2.53	8.60	1.51	29.70	2.67
1 week	4.60	0.52	3.75	0.79	52.25	2.51	6.80	1.23	32.70	2.46
Control	4.80	0.63	4.30	0.48	51.20	1.55	7.65	1.42	31.95	1.64
1 month	5.50	0.85	4.20	1.50	48.05*	1.04	8.30	0.95	33.45	1.57
Control	5.90	0.88	4.70	1.42	50.60	3.44	7.50	2.01	31.30	3.86
2 months	5.40	1.08	3.70	1.36	50.90	1.61	6.00	1.89	34.00	2.94
Control	5.95	0.76	4.10	1.29	50.85	6.41	7.40	1.15	31.90	4.18
3 months	5.65	1.00	3.80	1.30	49.07	3.20	6.55	1.46	35.20	4.29
Control	6.40	0.97	3.75	0.86	50.95	4.85	7.35	1.77	31.65	4.85

**t-test significantly different from control group at 5% level of significance.*
***t-test significantly different from control group at 1% level of significance.*
s = standard deviation.

Preliminary toxicological study of neem oil on laboratory mice

Many investigators have studied the toxicological effect (pathological, histological, pharmacotoxic, biochemical, cytotoxic, and reproductive and developmental toxicity) of natural extracts of neem or neem-based industrial formulations on various mammalian species [*8–15*].

In this study, neem oil was administered to laboratory mice for 90 days. There was no significant effect on weekly body weight gain, serum AST and ALT or blood parameters. Similarly, it was found that a technical 12% preparation of azadirachtin, when administered to male and female rats for 90 days did not produce any signs of toxicity, mortality, changes in tissue weight, pathology and serum and blood parameters [*8*]. However, dose-related pharmacotoxic symptoms were noted in another study when neem oil was orally administered to rats and rabbits [*9*].

Aqueous neem leaf extract when administered at 500 mg/kg body weight to rats with paracetamol-induced hepatotoxicity reduced the elevation of serum AST, ALT and GGT and also reduced liver necrosis [*12*]. Conversely, Vepacide® (a neem-based pesticide) administered orally to albino rats for 90 days caused significant elevation of AST and ALT in serum, kidney and lungs and these enzymes decreased in the liver in both sexes [*10*]. Margosa oil (neem oil) also caused great increase of blood AST and ALT and other blood constituents in male rats [*11*]. Administration of aqueous neem extract to rats for 10 weeks decreased total bilirubin in serum [*25*].

We conclude that neem oil could be used as a more environmentally friendly alternative to traditional larvicides.

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