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Larvicidal activities of chinaberry, neem and *Bacillus thuringiensis israelensis* (Bti) to an insecticide resistant population of *Anopheles arabiensis* from Tolay, Southwest Ethiopia

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ABSTRACT

Objective: To elucidate the larvicidal potency of neem, chinaberry and *Bacillus thuringiensis israelensis* (Bti) to larvae of *Anopheles arabiensis* under semi-field condition and adult susceptibility/resistance to the conventionally used insecticides in Tolay, Southwestern Ethiopia.

Methods: Wild collected 3rd and 4th stage larvae were exposed to neem, and chinaberry seed powder dissolved in water and Bti in artificial containers at three treatment levels: 0.2, 0.1 and 0.05 g/m^2 and controls were free of treatments. Larval and pupal mortalities were monitored daily and residual activities were determined. The experiments were replicated three times. The World Health Organization tube test for all classes of insecticides was conducted on adult *Anopheles arabiensis* reared from field collected larvae and pupae. Data were analyzed using STATA software version 11.

Results: In the first application, neem powder caused 88.9%, 87.9% and 79.4% larval and pupal mortality at 0.2, 0.1 and 0.05 g/m^2 after 4.3, 6.0 and 5.7 days, respectively. The corresponding killing effect of chinaberry was 80.3%, 62.1% and 30.3% after 7.0, 7.7 and 8.3 days respectively. Bti at all treatments killed 100% after 24 h except 2.7 days for 0.05 g/m^2. Adult mosquitoes were susceptible only for fenitrothion and pirimiphos-methyl with 100% mortality while resistant to deltamethrin, alpha-cypermethrin, etofenprox and dichlorodiphenyl-tricgloroethane with only 9.0%, 3.0%, 5.1% and 2.0% mortalities respectively.

Conclusions: Neem, chinaberry and Bti showed potent larvicidal and pupicidal activities. However, in the area, high level of mosquito resistance to pyrethroids and dichlorodiphenyl-tricgloroethane was seen which will pose serious challenge to vector control in the future. Therefore, using integrated approach including these botanical larvicides is warranted to manage insecticide resistance.

1. Introduction

Mosquito-borne diseases are significant contributors to disease burden, death, and poverty all over the world, particularly in tropical countries [1]. Among them, malaria which is caused by *Plasmodium* parasites remains the most serious disease [2]. Globally, an estimated 3.3 billion people are at risk of being infected and developing the disease. In 2013, an estimated 198 million cases of malaria occurred globally and the disease led to 584000 deaths. The burden is the heaviest in the World Health Organization (WHO) African Region, where about 90% of all malaria deaths occur, and in children aged less than 5 years, who account for 78% of all deaths [3].

Vector control, chemotherapy and early diagnosis are the main tools for the prevention and control of the disease. Insecticides are the most important elements in the integrated approach of vector control; however, many vector species of public health importance have already developed resistance to

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one or more insecticides [4]. Almost all public health insecticides are also used in agriculture and vectors may be exposed to the same or similar insecticidal compounds when they breed within or close to agricultural crops, which will select for resistance. This situation is of particular relevance for malaria vectors [2,4].

In Ethiopia, control of malaria depends on early diagnosis, effective treatment of patients and vector control. Vector control measures rely on selective indoor residual spraying (IRS), distribution of long lasting insecticide treated mosquito nets and source reduction of larval habitats. As a result, strong resistance of *Anopheles arabiensis* (*An. arabiensis*), the principal vector in the country, to insecticides has been reported [5,6].

Therefore, there is a need to find alternative control methods and evaluate their efficacy with the need to respond to the challenges of insecticide resistance. Larval control of malaria vectors is a well-proven preventive method that has been neglected, but deserves renewed consideration for malaria control programmes in the 21st century [7].

Although one organophosphate insecticide (temephos) and two bacterial origins [*Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus*] are utilized as larvicides, most agree to explore the potential larvicidal activity of botanicals such as neem and chinaberry to serve as alternatives [8]. Neem and chinaberry products contain multitudes of active ingredients with different modes of action, which lessens the chance of resistance developing in mosquito populations [9]. These plant based larvicides are also environmentally sound and locally accessible.

Therefore the main objective of the study was to evaluate the larvicidal potency of neem and chinaberry powders and Bti to larvae of *An. arabiensis* in Tolay, Southwest Ethiopia. In addition, the insecticide susceptibility status of *An. arabiensis* in the area was assessed.

2. Materials and methods

2.1. Study area

The study was conducted in Tolay/Wayu Wodeso Kebele (upper Ghibe Valley) in Chora Boter Woreda, which is located 243 km south west of Addis Ababa, at 80°14′ N, 37°35′ E. It is found at an altitude of 1 050–1 600 m above sea level with annual rainfall of 900–1 000 mm. Much of the annual rainfall is between June and August, and the dry season lasts from October to February. The maximum and minimum temperature in the area ranges between 30 and 37 °C and 10 and 15 °C, respectively. Malaria is the most prevalent disease in the area with a peak transmission period from October to November [10].

2.2. Study design and period

The study was done from September 2012 to January 2013 in Tolay, Oromia Regional State, South Western Ethiopia. Seed powders of neem and chinaberry and the bacterial larvicide, Bti, were evaluated against larvae of *An. arabiensis* under semi-field condition. Identification of test plants took place with the help of a botanist [Mr. Kassahun Mamo, International Center of Insect Physiology and Ecology (ICIPE)]. Chinaberry fruit was gathered from local trees growing in Bishoftu (Debre Zeit) town, South Eastern Ethiopia. This was done by simply collecting several kilograms of fruits from trees by picking low-hanging fruit from the trees.

Fruit was dried in shade and the flesh was removed by hand by squeezing each fruit and washed 4–5 times to avoid the sticky and mucoid part found immediately after the flesh. After drying the fruit for 6 days in the shade, it was crushed and powdered using wooden mortar and pestle. Then the powdered seed was stored in a dry, shaded plastic container for several days to use in field trials [11]. Neem seed was taken from ICIPE botanical laboratory which was collected from Dire Dawa in July 2012 and washed, dried and powdered. Bti was obtained from ICIPE laboratory which was in use for anopheline larval control in the breeding habitats in Tolay.

2.3. Larval collection and transportation

Second, third and fourth instar larvae of *An. arabiensis* were collected using WHO standard dippers from the natural breeding sites. Collection was done by randomizing the sites of larval habitat. The breeding sites which were used for collection include: small rain pools, hoof-prints, drains, ditches and streams from Gerengera River, Babo stream, broken pipe at the Military Camp and Degaga stream. Larvae were transported directly back to the ICIPE field laboratory in plastic jars. Larvae were placed into enamel plastic trays and were fed dry dog food. The tests were carried out on third and fourth instars while the second instar larvae were allowed to transform to the test stages. Larvae of *Anopheles gambiae* (*An. gambiae*) s.l. were differentiated by their shinny tergal plates and stout body in addition to their specific breeding sites such as small rain pools, hoof-prints, drains, ditches and streams. Some larvae from the sample were further identified morphologically by mounting on slides. Furthermore, the complex members were identified by PCR on adult samples reared from larvae taken from the same breeding habitats [12].

2.4. Experimental procedures under semi-field condition

Tests were conducted according to the methods of WHO, 2005 [13]. Plastic containers having an area of 100 cm^2 and more than 2 L capacity were used for the larvicidal bioassay in the field. The containers were half-buried in the ground, and 1 L (modified by adjusting prior to the tests) of water from the natural breeding habitats was added into each plastic container. Since this study used a granular seed powder instead of a laboratory derived solvent based solution, some of the procedures were adapted to allow for the use of seed powder in the field condition. The seed powder targets mosquito larvae because it floats on the water surface where larvae feed, so there was no effort to shake or stir to create a homogenous solution. The trials were done based on weights of dried seed per unit area of water surface on which the powdered seed was floated.

The trials were performed using graded bioassay with three treatment levels of neem and chinaberry seed powders and Bti [VectoBac water dispersible granule (WDG)] with a control group (without powder and Bti which is used as quality control according to WHO, 2005 guideline) for each type of treatment [11,13]. The three treatment levels were 0.05 g, 0.1 g and 0.2 g of powdered seed per 1 L was applied to each larval container [11]. For each concentration and the control, trials were done in three replicates.

The water-filled containers were given 24 h for conditioning or ageing, and then larvae were transferred from the plastic trays

to test containers using pasteur pipettes and larval food was added by broadcasting over the water surface [13]. For all treatment and control levels (0.05 g, 0.1 g, 0.2 g and control), 100 larvae were divided into four batches of 25 larvae and placed into identical larval containers. After 2–3 h of larval acclimatization, the containers were treated with selected dosages by spreading the powders over the water surface [13]. The containers were covered with nylon mesh screen to prevent other mosquitoes or insects from laying eggs, to protect the water from falling debris and also for containment of emerging adult mosquitoes. The water level in the containers was sustained by refilling every day [11,13]. Only larval food was given for all control groups. From the shape of the containers, the three treatment levels or doses were 0.05 g/m^2, 0.1 g/m^2 and 0.2 g/m^2. The temperature was checked at intervals for all containers [11]. Larval mortality was monitored every 24 h until no significance difference was observed between treatment and control. Dead larvae in all replicates were combined and expressed as a percentage of larval mortality in each concentration [13].

2.5. Residual test

The residual activities of the two botanicals and Bti against larvae were tested by adding another batch of 25 third and fourth instar larvae in treated and untreated (control) containers [13]. This was done when 100% of the larvae and pupae were either dead or some emerged as adults, in each replicate [13]. As with the initial batches of larvae, assessments of mortality were made every 24 h. The residual tests were repeated by introducing new batches of larvae starting from first to subsequent second, third and more applications until no significance difference in mortality was recorded between controls and treated batches [13]. After one batch of experiment was completed, the larval containers (pots) were cleaned and fresh batch of larvae were used for each replicate.

From these, the bio-efficacy of neem, chinaberry and Bti was determined. The results were also compared to verify which performs best in a given concentration and time. The residual impact of the products was also assessed.

2.6. Adult mosquito rearing and insecticide susceptibility tests

Larvae and pupae of *An. gambiae* s.l. (later identified with PCR) were collected from their natural breeding sites namely, Gerengera River, Degaga stream, and Babo sites and reared to adults at the ICIPE laboratory and Aklilu Lemma Institute of Pathobiology (ALIPB) insectary under standard conditions: (25 ± 2) °C temperature, 80% ± 4% relative humidity. Adults were fed on 10% sucrose solution. Two to three days old and non-blood fed females were exposed to WHO insecticide impregnated papers including dichloro-diphenyl-tricgloroethane (DDT), fenitrothion, pirimiphos-methyl, propoxur, bendiocarb, deltamethrin, alpha-cypermethrin and etofenprox. Each insecticide test was replicated four times containing 99–100 mosquitoes. The controls constituted mosquitoes exposed to oil impregnated papers (this was used to control the quality of the tests and to compare with insecticide impregnated papers and was according to WHO, 2013) [14]. All tests were conducted for 1 h with the exception of fenitrothion (2 h) and knockdown for DDT and the pyrethroid tests were recorded every 10 min [14,15]. At the end of the exposure period, mosquitoes were transferred to holding tubes and provided with 10% sucrose solution, held for 24 h after which mortality was recorded [15]. Finally interpretation of the results was made following the criteria given by WHO, 2013 [14].

2.7. PCR identification of An. gambiae

Samples of dead and surviving mosquitoes were preserved in 95% ethanol and kept in a freezer (−20 °C) for subsequent molecular identification. Leg segment of individual *An. gambiae* s.l. were taken and rDNA amplification was done by PCR technique to identify the sibling species of each mosquito [12].

2.8. Data analysis

The percentage mortality of larvae and pupae which was a measure of efficacy was calculated by using the following formula:

Percentage mortality in controls = number of dead larvae/number of larvae introduced × 100

Percentage mortality in experimental = number of dead larvae/number of larvae introduced × 100

When control mortality was between 5% and 20%, experimental mortality was corrected using Abbott's (1925) formula:

Corrected percentage mortality:

$$\text{Mortality } (\%) = \frac{Y - X}{100 - X} \times 100$$

where Y = percentage mortality in the treated sample and X = percentage mortality in the control.

After data were collected on appropriate formats, it was transferred to MS Excel Window and was analyzed by Stata Software Version 11. Two-sample test of proportion/significant difference test were used and 95% confidence intervals (*CI*) were calculated in order to compare the larvicidal potency of the plants and susceptibility of the test mosquito larvae. The values were judged as significantly different between the plant extracts when $P < 0.05$ if the *CI* did not overlap.

3. Results

3.1. PCR test result for species identification

Eighty three *An. gambiae* s.l. samples were assayed by PCR and all were identified as *An. arabiensis* with the exception of six specimens that were not identified despite two attempts at amplification. This may be due to a problem in DNA preservation and subsequent DNA degradation.

3.2. Larvicidal and pupicidal activities of chinaberry, neem and Bti

3.2.1. Bio-efficacy of chinaberry [Melia azedarach (M. azedarach)]

The bio-efficacy (larvicidal and pupicidal effect) of this plant powder in the first application was 80.3%, 62.1% and 30.3% at

0.2 g/m^2, 0.1 g/m^2 and 0.05 g/m^2, after 7.0, 7.7 and 8.3 days (Table 1). The larvicidal and pupicidal efficacy was higher at 0.2 g/m^2 than 0.1 g (P = 0.007) and 0.05 g/m^2 (P = 0.000). 0.1 g/m^2 was more effective (62.1% mortality) than 0.05 g/m^2 (30.3% mortality) (P = 0.000). In the second application of the residual tests, mortality rate was declining compared to the first application for the two doses. The two doses *i.e.* 0.2 and 0.1 g/m^2 showed similar killing effect with 56.3% mortality and 0.05 g/m^2 treatment level showed 16.9% mortality. In the third application of the residual test, mortality for all treatment doses was not significantly different from the control mortality and the experiment was discontinued. The respective residual time (in days) taken for 0.2 g/m^2, 0.1 g/m^2 and 0.05 g/m^2 treatments were 12.7 days (56.3% mortality), 13.0 days (56.3% mortality) and 8.3 days (30.3% mortality) respectively.

3.2.2. Bio-efficacy of neem [Azadirachta indica (A. indica)]

The bioefficacy of neem seed powder on *An. arabiensis* larvae and pupae in the first application at 0.2 g/m^2, 0.1 g/m^2 and 0.05 g/m^2 treatment levels were 88.9%, 87.3% and 79.4%, after 4.3, 6.0 and 5.7 days respectively (Table 2). There was no significant difference between the higher dose (0.2 g/m^2) and the two lower treatment levels (0.1 g/m^2 and 0.05 g/m^2) in the first application (P = 0.76 and 0.1). But in the second application, 0.2 g/m^2 treatment level showed high larval and pupal mortality (78.5%) than 0.1 g/m^2 (50.8%, P = 0.00) and 0.05 g/m^2 (20.0%, P = 0.00) treatment levels. In the third application, only 0.2 g/m^2 showed significant effect with 59.4% mortality.

In these successive residual tests, the persistency (bio-efficacy) was decreased as the time and numbers of applications were increased. At 0.2 g/m^2, 59.4% mortality was seen up to 15 days of the residual tests which was the longest time.

3.2.3. Effect of Bti

Larvae of *An. arabiensis* were highly susceptible to Bti, with 100% mortality at all the three doses of treatment levels in the first application of the trials (Table 3). The formulations provided 100% larval mortality within 24 h at 0.2 g/m^2 and 0.1 g/m^2 and within about 3 days at 0.05 g/m^2. In the second application of the residual test, 0.2 g/m^2 dose showed 100% mortality within about 3 days which was significantly different from 0.1 g/m^2 (69.7%, P = 0.00) and 0.05 g/m^2 (78.8%, P = 0.00). In general, percentage mortality of larvae and pupae was getting low in the consecutive tests of consistency. A prolonged residual effect was seen at 0.2 g/m^2 concentration and lasted up to 13 days to kill about 50% of the larval population.

3.3. Comparative larvicidal potency of chinaberry and neem

In their higher dose (0.2 g/m^2), chinaberry and neem showed larval mortality of 80.3% and 88.9%, respectively in the first application. But there was no significant bio-efficacy difference between the two plant powders (P = 0.15) at this application.

But from the entire tests, neem seed powder had higher effect than chinaberry in the residual tests and lower doses of the treatments (Tables 1 and 2).

3.4. Bio efficacy of chinaberry and neem relative to Bti

At all the treatment doses, chinaberry and neem powders showed a relatively lower efficacy than Bti on *An. arabiensis* larval and pupal mortality (Tables 1–3). This effect was seen throughout the residual tests. But, the residual time (persistency) taken to finish their killing capacity did not show much difference with 13 days, 15 days and 13 days for chinaberry, neem and Bti, respectively.

3.5. Adult insecticide susceptibility test results

A total of 798 *An. arabiensis* were tested for susceptibility against insecticides. A minimum of 99 and a maximum of 100 mosquitoes were exposed to each of the insecticides. The

Table 1

Bio-efficacy of chinaberry (*M. azedarach*) seed powder on larval and pupal mortality of *An. arabiensis* in Tolay, South Western Ethiopia.

Dose of Rx (g/m^2)	Mean larval and pupal mortality (%)					
	1st larval application[*,#]	P-value	2nd larval application[*,#]	P-value	3rd larval application[*,#]	P-value
0.2	80.3 (0.5667–0.8092)	0.000	56.3 (0.3869–0.6333)	0.000	9.4 (0.0508–0.1566)	0.840
0.1	62.1 (0.3690–0.6333)	0.000	56.3 (0.3869–0.6333)	0.000	14.0 (0.1060–0.1182)	0.542
0.05	30.3 (0.0556–0.3104)	0.003	16.9 (0.0168–0.2146)	0.012	16.0 (0.1438–0.1838)	0.403
Control	12.0		5.3		14.7	

[*]: Data in parentheses are 95% *CI* which are calculated relative to the control (from mean difference of treatment and control); [#]: Values are the mean percentages of three replications; Rx: Treatment.

Table 2

Bio-efficacy of neem (*A. indica*) seed powder on larval and pupal mortality of *An. arabiensis* under semi-field condition in Tolay, South Western Ethiopia.

Dose of Rx (g/m^2)	Mean larval and pupal mortality (%)							
	1st larval application[*,#]	P-value	2nd larval application[*,#]	P-value	3rd larval application[*,#]	P-value	4th larval application[*,#]	P-value
0.2	88.9 (0.6197–0.8382)	0.000	78.5 (0.5305–0.7720)	0.000	59.4 (0.3100–0.5840)	0.004	26.2 (0.0568–0.2606)	0.105
0.1	87.3 (0.6009–0.8250)	0.000	50.8 (0.2375–0.5112)	0.000	9.4 (0.1568–0.0508)	0.319	–	–
0.05	79.4 (0.5104–0.7575)	0.000	20.0 (0.0521–0.1854)	0.136	4.7 (0.1933–0.0066)	0.980	–	–
Control	16.0		13.3		14.7			

[*]: Data in parentheses are 95% *CI* which are calculated relative to the control (from mean difference of treatment and control); [#]: Values are the mean percentages of three replications; Rx: Treatment.

Table 3

Susceptibility of *An. arabiensis* larvae and pupae to Bti under semi-field condition in Tolay, South Western Ethiopia.

Dose of Rx (g/m^2)	Mean larval and pupal mortality (%)									
	1st larval application*,#	*P*-value	2nd larval application*,#	*P*-value	3rd larval application*,#	*P*-value	4th larval application*,#	*P*-value	5th larval application*,#	*P*-value
0.2	100	0.000	100	0.000	72.0 (0.4861–0.7338)	0.000	50.0 (0.3029–0.5570)	0.000	32.0 (0.1397–0.4202)	0.000
0.1	100	0.000	69.7 (0.4844–0.7295)	0.000	43.8 (0.1952–0.4607)	0.000	10.3 (0.0568–0.1228)	0.472		
0.05	100	0.000	78.8 (0.5850–0.8109)	0.000	34.4 (0.1052–0.3627)	0.001	17.4 (7.5500–0.2066)	0.053		
Control	0		9		11		7			

*: Data in parentheses are 95% *CI* which are calculated relative to the control (from mean difference of treatment and control); #: Values are the mean percentages of three replications; Rx: Treatment.

mortality rate in all the control populations were < 5%, therefore, Abbott's correction was not necessary during data analysis. *An. arabiensis* was susceptible to the organophosphates, pirimiphos methyl and fenitrothion with 100% mortality (Table 4). It also exhibited an intermediary level of resistance to the two carbamates – propoxur and bendiocarb with 90.0% and 92.9% mortalities. Lower mortality was observed when mosquitoes were exposed to DDT, deltamethrin, alpha-cypermethrin and etofenprox showing confirmed resistance (Table 4).

Table 4

Insecticide susceptibility/resistance status of *An. arabiensis* from Tolay.

Insecticide	No.	Mortality (%)	Susceptibility status	Resistance ratio (MR)
DDT	100	2.0	R	0.02
Permiphos methyl	100	100.0	S	1.00
Fenitrothion	100	100.0	S	1.00
Propoxur	100	90.0	SR	0.90
Bendiocarb	99	92.9	SR	0.93
Deltamethrin	100	9.0	R	0.09
Alpha-cypermethrin	100	3.0	R	0.03
Etofenprox	99	5.1	R	0.05

No.: Total number of mosquitoes exposed to each insecticide; S: Susceptible; R: Resistance; SR: Sign of resistance; MR: Mortality ratio (% mortality of *An. arabiensis*/% mortality of *An. arabiensis* from ALIPB colony).

The KT50 and KT90 values for DDT, deltamethrin, alpha-cypermethrin, and etofenprox were not calculated because they did not show more than 50% mortality on the mosquito populations after 24 h exposure time. The number of mosquitoes knocked down after 60 min exposure times were only six for deltamethrin and alpha-cypermethrin each but nil for DDT and etofenprox (Table 5).

Table 5

Number of *An. arabiensis* knocked down after 60 min of exposure.

Insecticide	No. of knockdown
DDT (4%)	0
Deltamethrin (0.05%)	6
Alpha-cypermethrin (0.05%)	6
Etofenprox (0.5%)	0

An. arabiensis from an insectary colony showed 100% mortality to the insecticides tested and the resistance ratio was very high indicating the severity of resistance in the wild colonies (Table 4).

4. Discussion

In the current study, the crude extract of seed powder was used. This is advantageous because, the crude extracts of plants may be more effective compared to the individual active compounds, due to natural synergism that discourages the development of resistance in the vectors [16]. Insects also took longer to develop resistance to a mixture of natural active ingredients than to any of separate components [17].

The efficacy of chinaberry seed powder against larvae and pupae of *An. arabiensis* in the first application was 80.3%, 62.1% and 30.3% at 0.2 g/m^2, 0.1 g/m^2 and 0.05 g/m^2. However, a similar study by Trudel and Bomblies [11] in Asendabo, Ethiopia, showed higher efficacy with 100% mortality at 0.2 g/m^2 and 0.1 g/m^2 and 93% mortality at 0.05 g/m^2. The reasons for the lower values in the present study might be due to the setup of the experiment, since it was conducted under semi-field condition, while the study by Trudel and Bomblies [11] was in controlled, idealized laboratory setting. The field study may be partly exposed to wind gusts blowing the powdered seeds on the surface of pots to one side of the pots, leaving areas of pot surface without seed powder. The efficacy in the field may also be lower than in the laboratory due to exposure of chemicals to radiation which may cause degradation of biologically active compounds with larvicidal effect. In laboratory tests, Trudel and Bomblies [11] used 333 mL water, but due to high rate of evaporation in the present study area, 1 L of water was used with 3 cm depth. As the dose of chinaberry increased, the efficacy was also shown to be increased. This was in agreement with Trudel and Bomblies [11], which showed higher mortality at higher doses against *An. arabiensis* larvae. Similarly, an increase in concentration of different extracts showed continuous increase in mortality of 3rd and 4th instar larvae against *Culex quinquefasciatus* (*Cx. quinquefasciatus*) and in all stages of *An. stephensi* [18,19]. In the present study, mortality declined as the residual time was prolonged with 56.3% mortality for 0.2 and 0.1 g treatments and only 16.9% mortality for 0.05 g treatment in the second application of the residual test. The longest residual time was about 13 days at the two higher doses which killed about 56.3% of larvae and pupae and only 30.3% were killed at 0.05 g/m^2 within 8 days. This shows the consistency of the plant powder lasting for about 2 weeks killing substantial number of larvae and pupae of *An. arabiensis* at its higher doses (especially at 0.2 g/m^2).

The larvicidal effect of this plant could be due to the presence of limonoids which exhibit anti-feedent property that lead to killing of insect larvae [20,21]. They also possess poisonous effect

on insects and destroy the structure of integument and the alimentary canal causing disorganization of the extracellular membrane layers and the basal portion of the epithelial cells of the stomach [22,23].

Neem in the present study was shown to be potent against larvae and pupae of *An. arabiensis*. High rates of larval and pupal mortality were observed at all concentrations. In the first few days of the tests, lower doses (0.05 g and 0.1 g) had similar killing efficacy with the higher dose (0.2 g) of neem seed powder. But in the following tests of consistency, only 0.2 g treatment level showed significant larval and pupal mortality which indicated the dose response relation *i.e.* the highest dose is more potent and has longer residual activity with about 2 weeks duration to kill about 59.4% of larvae and pupae. This could be due to presence of more amounts of active compounds with insecticidal activity. Higher concentrations of neem oil formulation caused higher mortality against *An. gambiae* s.s. larvae, while at lower concentrations the rate of mortality was very low [9]. Larval mortality of *An. stephensi*, when exposed to different concentrations of neem seed kernel extract, was increased as the concentration was increasing [24], and methanolic extract of neem seed kernel caused 100% larval mortality within 12 h at highest concentration [25]. These studies support the dose response relations of neem seed powder in our study even though the species of mosquito and the formulation are different.

There are various reports on the insecticidal properties of neem from trials conducted under field conditions. A recent study revealed that, application of neem oil formulation in different breeding sites under natural field conditions provided 98.1% reduction of *Anopheles*. All these are comparable with the efficacy of neem in our study under semi-field condition [8].

Although, comparison of results of the present study with the outcomes of various other studies on the efficacy of different neem products is difficult, our study shows a potent larvicidal and pupicidal efficacy against *An. arabiensis*. There are numerous differences with the previous studies, notably differences in the origin of products, concentrations of active ingredients of the products, the species of mosquitoes tested, modes of application of the products, experimental setup and parts of the neem plant from which the products were extracted.

In this study, the larvicidal and pupicidal efficacy of neem seed powder could be due to compounds found in neem seed extract, especially azadirachtin found in higher concentrations. Besides azadirachtin, there are other triterpenoids such as salanine, meliantrol and nimbina, which are the most significant ones, since they have proven their ability to inhibit the growth of pest insects of both agricultural and human health importance [23].

Azadirachtin can be found in the bark, leaves, fruits and mostly in seeds of the neem tree [26]. It has two profound effects on insects: at the physiological level by blocking the synthesis and release of molting hormones (ecdysteroids) from the prothoracic gland, leading to incomplete ecdysis in immature insects and it is a potent antifeedant to many insects [27].

Comparing larvicidal potency of chinaberry and neem at their higher dose (0.2 g), they showed equivalent effect at the beginning of the residual test (in the first larval application) but from the entire tests, neem seed powder had higher effect than chinaberry in the residual tests and at the lower doses of the treatments. This could be due to differences in the azadirachtin content and other compounds.

Our result also showed that *An. arabiensis* is highly susceptible to Bti WDG (VectorBac, 3000 ITU/mg) under semi-field conditions. This is comparable with a study in Gambia which showed 95% mortality on 3rd stage larvae of *An. gambiae* s.l. and *An. gambiae* s.s. at 0.132 mg/L after 24 h exposure [28].

Similar to our study, commercial formulations of Bti VectoBac showed 100% mortality within 48 h against *Anopheles claviger* and *Aedes cantans* in the laboratory and under semi-field conditions in Sudan and it was effective against *An. arabiensis* and *Cx. quinquefasciatus* [29]. Another research on Bti also showed up to 100% mortality within 24 h against *An. gambiae* s.l. larvae and showed an overall reduction in mosquito emergence with good effect at lowering pupal populations which is in line with the current study [30]. This microbial larviciding also reduced *Anopheles* larval density in rural Kenya [31]. Bti also caused significant mortality of *An. arabiensis*, *Anopheles cinereus*, *Anopheles pretoriensis* and *Cx. quinquefasciatus* in Eritrea [32].

The residual activity of Bti in the present study was about 13 days at the higher dose (0.2 g) of treatment which is comparable with result in Sudan which was 15 days against *An. arabiensis* and *Cx. quinquefasciatus* [29]. However, there was lower residual effect, about10 days, in standardized field tests to *An. gambiae* s.l. carried out during the dry season in the Gambia [27]. Very low residual activity of Bti was reported against *An. gambiae* s.l. with residual activity of only 2–3 days after treatment and indicated quick and continuing re-colonization of all treated sites by early instars [30]. These differences with our study could be due to the difference in the species of *Anopheles* mosquito tested.

Bti has high larvicidal activity due to toxicity of the spore crystal complex which is a synergistic interaction between the 25 kDa protein and other proteins. When the spore-crystal of Bti containing toxic proteins (protoxins) is ingested by larvae of *An. arabiensis*, the pro-toxins are solubilized in alkaline pH of the larval gut and get activated in the form of toxins leading to death [33].

An. arabiensis showed extremely high level of resistance in Tolay to the organochlorine (DDT, 2% mortality) and pyrethroids (9% for deltamethrin, 3% for alpha-cypermethrin, and 5.1% for etofenprox) and an intermediary level of resistance to be confirmed for the two carbamates, propoxur and bendiocarb with 90.0% and 92.9% mortalities, respectively. However, susceptibility to the two organophosphates, permiphos-methyl and fenitrothion with 100% mortality was maintained.

The susceptible status of laboratory colonies from ALIPB insectary were more susceptible with 0.98 times (49 fold) for DDT, 0.91 times (10 fold) for deltamethrin, 0.97 times (32.3 fold) for alpha-cypermethrin and 0.95 times (19 fold) for etofenprox when compared with wild species of *An. arabiensis* which shows the extensive resistance of these mosquito population in Tolay. This high resistance to DDT is in agreement with Yewhalaw *et al.* [6] who reported high level of resistance (1.0% mortality) on *An. arabiensis* from South Western Ethiopia. The study also showed resistance to deltamethrin (82.2%) as has been observed in our study. There is wide distribution of *An. arabiensis* resistance to DDT and deltamethrin in different parts of Ethiopia including in the study area. DDT and deltamethrin resistance in Ghibe River Valley and DDT resistance in Gorgora villages and DDT and deltamethrin resistance in different localities in the country

are consistent with our findings [5,34]. There was also DDT and permethrin resistance in Eastern and Central Sudan and partial resistance to permethrin from Lower Moshi, Estern Tanzania [35,36]. Our study shows a sharp contrast with the study in Mwea, Western Kenya which showed 100% mortality to DDT and permethrin and 99.46% to lambdacylothrin, as well as 99.8% to deltamethrin in Khartoum City, Sudan [37,38], which could be attributed to long intensive use of pyrethroids for IRS and long lasting insecticide treated mosquito nets in the study area and in the country as a whole and due to previous use of DDT for long time which may cause cross resistance. In line with our study, partial resistance to bendiocarb was reported in Northern Ethiopia [39]. However, a report on susceptibility of *An. arabiensis* to bendiocarb (98.1% mortality) and propoxur (100% mortality) in Sudan disagree with our results which showed 92.9% mortality for bendiocarb and 90% mortality for propoxur [38], even though it was consistent to the result of fenitrothion (100% mortality) in our study.

This study showed that, chinaberry and neem seed powders are effective larvicides under semi-field condition against *An. arabiensis*, the principal malaria vector in Tolay, South Western Ethiopia. From the entire tests, higher doses of the plants seed powder showed higher larvicidal efficacy, even though neem seed powder showed similar efficacy at lower doses in the first few days of the residual tests. Comparing the larvicidal efficacy of these two plants, neem seed powder was shown to be better larvicide against *An. arabiensis* larvae and pupae. Although these methods will not replace currently employed malaria control strategies (IRS and insecticide-treated nets, both relies on synthetic insecticides), the seed powder of neem and chinaberry which are environmentally safe and eco-friendly could be additional tools to be used in an integrated approach to fight malaria sustainably.

Larvae of *An. arabiensis* were shown to be highly susceptible to Bti, VectoBac, WDG under semi-field condition even at the lower dose of treatment level (0.1 g/m^2 to kill 100% of the larvae within 24 h). Bti was shown to have more efficacies than both botanical products (chinaberry and neem), however, their residual activity was more or less similar.

This is the first semi-field trial on powdered form of seed of neem and chinaberry on the larvicidal potency against *An. arabiensis* in Ethiopia. Therefore, the result of this research can be used as baseline for large scale field experiment. The public can benefit by using these eco-friendly and easily accessible plant products to control malaria vectors. In Tolay area, where malaria is known to be prevalent, ICIPE is conducting different researches on environmentally sound larval control measures. The current study, therefore, will support the program by providing base line information on the comparative larvicidal potency of neem and chinaberry seed powder as well as the susceptibility of larvae of *An. arabiensis* to Bti.

The result of this study clearly indicated that, high insecticide resistance of *An. arabiensis* for organochlorine (DDT) and pyrethroids (deltamethrin, alpha-cypermethrin and etofenprox) is widely spread in Tolay, which could greatly affect malaria vector control in the area as well as in the country. Sign of resistance to be confirmed was also seen on carbamates (bendiocarb and propoxur) indicating resistance of the vector to almost all classes of insecticides which will make vector control, a very complex problem in the country.

Conflict of interest statement

We declare that we have no conflict of interest.

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