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# Growth inhibitory effect of phenolic extracts of *Ziziphus jujuba* Mill. in dengue vector *Aedes aegypti* (L) in parent and F1 generation

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ABSTRACT

**Objective:** To evaluate the lethal and emergence inhibitory effect of alkaloid, phenolic and terpenoid extracts of *Ziziphus jujuba* (Rhamnaceae) against *Aedes aegypti* (Diptera: Culicidae), and to explore the effect of the most effective fraction on developmental and biochemical parameters of the dengue vector.

**Methods:** The fourth instar larvae of *Ae. aegypti* were exposed to alkaloid, phenolic and terpenoid extracts from *Z. jujuba* leaves to test their toxicity and emergence inhibitory effects. Phenolic extract, being the most effective was further tested against the mosquitoes for their growth inhibitory effect supported by biochemical changes in the parent and F1 generation.

**Results:** While the different secondary metabolite fractions *i.e.*, alkaloid, phenolics and terpenoid caused mortality at larval and pupal stages, the $LC_{50}$ value was the lowest for phenolic fraction. Further study carried out with the phenolic fraction revealed that it affected growth by decreasing adult life span, fertility and fecundity of the mosquitoes. The reduction in growth was also accompanied by decrease in carbohydrate and lipid levels.

**Conclusions:** It is concluded that the phenolic extract of the leaves of *Z. jujuba* is a potential candidate for control of *Aedes* mosquitoes.

## 1. Introduction

A wide selection of plants from herbs, shrubs and large trees have been extracted for their insecticidal properties [1]. Many of the plant secondary metabolites having pesticidal properties serve as larvicide, adulticide, antifeedant, insect growth regulators, repellents and attractant [2,3]. They affect insect physiology in many different ways and through various receptors which reduce the chances of developing resistance against them [1,4]. Terpenes constitute the largest group of plant based natural products and are reported to synergize the effects of other toxins by facilitating their passage through membranes. Similarly, alkaloids and phenolics are also reported to have insecticidal toxicity.

The efficacy of phytochemicals in mosquito control is extensively studied by recent workers. Members of the plant families including Solanaceae, Asteraceae, Euphorbiaceae, Moraceae, Myrtaceae, Cladophoraceae, Lamiaceae, Labiatae, Miliaceae, Oocystaceae, Leguminosae, Zingiberaceae, Rutaceae, *etc.* have various types of larval, adulticidal or repellent activities against different species of mosquitoes [1,5]. But studies related to their effect on physiology of the vectors are comparatively less although the knowledge may contribute in understanding the mechanism of action of the toxic compound(s).

Therefore, we focused on the effects of alkaloids, phenolics and terpenoids from *Ziziphus jujuba (Z. jujuba)* (Rhamnaceae) on the developmental and biochemical profile of dengue vector, *Aedes aegypti (Ae. aegypti)* in addition to their effect on mortality.

## 2. Materials and methods

### 2.1. Biological material

#### 2.1.1. Plant material collection and extraction

*Z. jujuba* leaves were collected from different locality of Dibrugarh district of Assam, India. The collected plants were

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shade dried, ground into a homogenous coarse powder and extracted with ethanol. The extracts were concentrated in rotaevaporator (IKON Instruments, Delhi) and the residue obtained was used for larvicidal assay. The extracts were further fractionated into alkaloids, phenols and terpenoids by following Harborne [6] and used for studying their effects on developmental and biochemical parameters.

#### 2.1.2. Mosquito collection

Eggs of *Ae. aegypti* were kindly provided by the Regional Medical Research Centre, Dibrugarh. Eggs were hatched by keeping in container filled with tap water and larvae were fed on a diet composed of yeast powder and dog biscuit.

### 2.2. Bioassay

#### 2.2.1. Larvicidal assay

A total of 0.1 g extracts was initially mixed with 50 μL of ethanol and then dissolved in 100 mL distilled water. A total of 10 μL of Tween 20 was added to the solution. The stock solution of 1 000 ppm was serially diluted to obtain different concentrations and 5 μL of Tween 20 was added to the solution after each dilution. The control was set up with 50 μL of ethanol, 10 μL of Tween 20 and 100 mL of distilled water.

For larvicidal assay, larvae were treated with seven concentrations ranging from 1000 ppm to 15.625 ppm. Five replicates were maintained each containing 20 insects. The mortality was recorded after 24 h of treatment and the data were subjected to statistical analysis for further study.

#### 2.2.2. Emergence inhibition assay

The assay was conducted using the results obtained in the above mentioned larvicidal assay. Early 4th instar larvae were exposed to lethal concentration causing 50% mortality ($LC_{50}$), lethal concentration causing 40% mortality ($LC_{40}$), lethal concentration causing 30% mortality ($LC_{30}$), lethal concentration causing 20% mortality ($LC_{20}$) and lethal concentration causing 10% mortality ($LC_{10}$) of each fraction for 24 h and then transferred to normal water. Development was allowed to continue until adult emergence. The total number of adults emerging after treatment was recorded and emergence inhibition concentration that inhibit 50% adult emergence ($EI_{50}$) was calculated. $EI_{50}$ denotes the concentration that causes 50% emergence inhibition.

#### 2.2.3. Effect on larval development and fertility

Five replicates of early 4th instar larvae each containing 20 larvae were exposed to $EI_{50}$ concentration. After 24 h of exposure, larvae were transferred to clean water and development was allowed to continue. Randomly selected males and females exposed to treatment were caged together and allowed to couple. Eggs collected were developed as F1 generation.

To determine the effect of the extracts on growth and development of larvae in parent and F1 generation the following parameters were considered:

Mortality: Number of dead larvae and pupae were counted at an interval of 24 h until emergence. Adults failing to survive up to 24 h post emergence were counted for adult mortality.

Growth period: For the parent generation, the growth period of 4th instar larvae and pupae was considered whereas for the F1 generation, growth period of the early instars was also recorded. Growth period was counted as the number of days between hatching/occurrence of one moult and completion of the succeeding moult.

Malformation: For the malformation count, number of deformed larvae, pupae and adult was considered for both parent and F1 generation. Malformation included incomplete moulting, presence of larval-pupal or pupal-adult intermediates and lack of melanisation.

Adult longevity: Average adult life span was counted in both parent and F1 generation.

Fecundity: For the fecundity assay, 4–5 d old mated females were blood fed and allowed to lay eggs in individual paper cups. The number of eggs produced was counted for a period of 7 d.

Fertility: hatching of eggs from treated females was allowed after 10 d of laying and the number of larvae hatched was counted for 48 h.

#### 2.2.4. Biochemical estimation

Larvae were treated with $EI_{50}$ concentration for 24 h and were then allowed to develop. Estimations of total protein, lipid and carbohydrate levels were done in late 4th instar larvae and 1 day old pupae in both parent and F1 generation.

Tissues for biochemical estimation were extracted following the protocol of Handel [7,8]. Total protein was estimated by the method of Lowry *et al* [9] and the total lipids and carbohydrates were estimated by the method of Handel [7,8].

### 2.3. Statistical analysis

The data was subjected to Probit analysis to calculate $LC_{50}$ and $EI_{50}$ after exposure to the extracts. The results of the different treatments were compared by independent sample *t*-test. All the calculations were carried out using SPSS 17 software package.

## 3. Results

### 3.1. Toxicity and emergence inhibition

The toxicity of *Z. jujuba* leaf secondary metabolites against 4th instar larvae of *Ae. aegypti* after 24 h of exposure is shown in Table 1. Phenolic extracts showed highest efficacy followed by terpenoid extracts. Alkaloid extracts showed lowest efficacy.

Table 1 also shows the emergence inhibition activity of the secondary metabolites on larvae exposed to sublethal concentrations. Early 4th instar larvae were exposed to concentrations causing 50%, 40%, 30%, 20% and 10% mortality.

### 3.2. Effect on larval development and fertility

Early 4th instar larvae were treated with 34.26 ppm phenolic extract ($EI_{50}$) and the different effects are listed in Tables 2 and 3. Table 2 shows percentage mortality and percentage malformation of different developmental stages against treatment with $EI_{50}$. In the parent generation, very low percentage mortality of 1.33% was seen in the 4th instar larvae after treatment. But higher mortality of 30% and 62% was seen in pupae and adult respectively after treatment with the phenolic extracts. In the F1 generation too, low larval mortality was recorded in the 3rd and 4th instars while 25% and 50% mortality was recorded in the pupal and adult stages respectively. No mortality was recorded in the control insects of both the generations. Very low

**Table 1**

$LC_{50}$ and $EI_{50}$ activity of alkaloid, phenolic and terpenoid extracts of *Z. jujuba* against 4th instar larvae of *Ae. aegypti* after 24 h of exposure.

| Extracts | $LC_{50}$ (in ppm) (95% *CI*) | Regression equation | $EI_{50}$ (in ppm) (95% *CI*) | Regression equation |
|---|---|---|---|---|
| Alkaloid | 320.20 (242.64–446.80) | −20.57 + 8.57*x* | 241.68 (190.94–266.42) | −23.70 + 10.00*x* |
| Phenol | 41.45 (35.18–49.00) | −6.21 + 4.28*x* | 34.26 (29.95–43.39) | −6.47 + 4.28*x* |
| Terpenoid | 189.67 (165.94–216.68) | −19.07 + 8.57*x* | 183.35 (169.77–219.31) | −22.20 + 10.00*x* |

**Table 2**

Total percentage mortality and percentage malformation of three replicates of 50 *Ae. aegypti* in both parent and F1 generation after treatment with $EI_{50}$ concentration of phenolic extract of *Z. jujuba*.

| Type | | % mortality | | % malformation | |
|---|---|---|---|---|---|
| | | Treatment group | Control group | Treatment group | Control group |
| Parent generation | 4th instar larvae | 1.33 | 0.00 | 0.00 | 0.00 |
| | Pupae | 30.00 | 0.00 | 10.00 | 0.00 |
| | Adult | 62.00 | 0.00 | 6.00 | 0.00 |
| F1 generation | 1st larvae | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2nd larvae | 0.00 | 0.00 | 0.00 | 0.00 |
| | 3rd larvae | 7.33 | 0.00 | 0.00 | 0.00 |
| | 4th larvae | 9.34 | 0.00 | 0.00 | 0.00 |
| | Pupae | 25.34 | 0.00 | 2.67 | 0.00 |
| | Adult | 50.67 | 0.00 | 0.00 | 0.00 |

**Table 3**

Effect of phenolic extracts of *Z. jujuba* on growth period, fecundity and percentage of hatching of *Ae. aegypti*.

| Items | | | Phenolic extract group | Control group |
|---|---|---|---|---|
| Parent generation | Growth period (No. of days) | 4th instar larvae | 3.34 ± 0.28 | 3.00 ± 0.50 |
| | | Pupae | 3.00 ± 0.50 | 2.50 ± 0.50 |
| | | Adult | 7.50 ± 1.73* | 27.00 ± 1.32 |
| | Fecundity | | 61.67 ± 2.52* | 98.00 ± 3.00 |
| | % Hatching | | 33.34 ± 11.54* | 100.00 ± 0.00 |
| F1 generation | Growth period (No. of days) | 1st instar larvae | 2.00 ± 0.50* | 1.00 ± 0.00 |
| | | 2nd instar larvae | 1.83 ± 0.57 | 1.00 ± 0.00 |
| | | 3rd instar larvae | 1.83 ± 0.28 | 1.50 ± 0.50 |
| | | 4th instar larvae | 4.00 ± 0.50 | 3.00 ± 0.50 |
| | | Pupae | 2.50 ± 0.00 | 2.50 ± 0.50 |
| | | Adult | 13.83 ± 0.76* | 27.00 ± 1.32 |
| | Fecundity | | 62.67 ± 2.08* | 96.34 ± 1.53 |
| | % Hatching | | 56.67 ± 5.77* | 100.00 ± 0.00 |

*Significantly different from control, $P < 0.05$.

percentage malformations were recorded in the pupae and adult of both generations after treatment with the phenolic extracts.

The effect of the phenolic extract of *Z. jujuba* on the growth period, fecundity and fertility of the treated mosquitoes is represented in Table 3. The adult life span of the treated mosquitoes of the parent generation significantly decreased ($t$ = 15.497, $P < 0.05$). In the F1 generation too, the adult life span showed significant decrease ($t$ = 14.930, $P < 0.05$). Significant decrease in fecundity post treatment was observed in both parent (t = 16.071, $P < 0.05$) and F1 generation (t = 22.584, $P < 0.05$). The percentage hatchability of the eggs laid by the treated females also decreased significantly in both generations ($t$ = 10.00, $P < 0.05$ in parent; $t$ = 13.00, $P < 0.05$ in F1 generation).

### 3.3. Changes in the biochemical profile

The protein level increased while the lipid, glucose and glycogen level decreased significantly after treatment with the phenolic extracts of *Z. jujuba* (Table 4). In the larvae, protein level significantly increased ($t$ = 15.442, $df$ = 8 in parent;

**Table 4**

Changes in the protein, lipid, glucose and glycogen profile of *Ae. aegypti* larvae and pupae after treatment with phenolic extracts from *Ziziphus jujube* (μg/mg).

| Groups | | Protein | Lipid | Glucose | Glycogen |
|---|---|---|---|---|---|
| Larvae | Parent | 75.24 ± 2.40$^{b}$ | 37.31 ± 5.04$^{b}$ | 34.39 ± 2.33$^{b}$ | 27.02 ± 2.79$^{b}$ |
| | F1 larvae | 56.48 ± 4.23$^{c}$ | 35.10 ± 0.97$^{b}$ | 18.26 ± 0.93$^{c}$ | 30.98 ± 0.86$^{c}$ |
| | Control | 31.13 ± 1.53$^{a}$ | 53.77 ± 4.34$^{a}$ | 55.42 ± 7.57$^{a}$ | 43.57 ± 1.09$^{a}$ |
| Pupae | Parent | 60.33 ± 9.62$^{b}$ | 35.84 ± 1.82$^{b}$ | 18.44 ± 1.03$^{a}$ | 29.19 ± 1.31$^{b}$ |
| | F1 pupae | 58.15 ± 0.13$^{b}$ | 29.23 ± 0.70$^{c}$ | 13.33 ± 0.72$^{b}$ | 26.68 ± 0.89$^{c}$ |
| | Control | 45.02 ± 2.99$^{a}$ | 40.82 ± 1.02$^{a}$ | 19.33 ± 1.31$^{a}$ | 37.72 ± 1.58$^{a}$ |

Different letters are significantly different from each other, $P < 0.05$.

$t$ = 10.797, $df$ = 8 in F1 generation). Significant decrease was seen in the lipid ($t$ = 5.527, $df$ = 8 in parent; $t$ = 9.367, $df$ = 8 in F1 generation); glucose ($t$ = 5.931, $df$ = 8 in parent; $t$ = 10.885, $df$ = 8 in F1 generation) and glycogen levels ($t$ = 12.326, $df$ = 8 in parent; $t$ = 20.163, $df$ = 8 in F1 generation) of larvae of parent and F1 generation respectively. Significant decrease was also recorded in the lipid ($t$ = 5.316, $df$ = 8 in parent; $t$ = 20.883, $df$ = 8 in F1 generation) and glycogen levels ($t$ = 9.250, $df$ = 8 in parent; $t$ = 13.552, $df$ = 8 in F1 generation) of pupae of both parent and F1 generation after treatment with the phenolic extracts. The protein level further decreased significantly in F1 generation as compared to the parent generation.

## 4. Discussion

From our study carried out to determine the effects of secondary metabolites of *Z. jujuba* leaves on *Ae. aegypti*, we report larvicidal efficacy of the alkaloid, phenolic and terpenoid extracts in a dose dependent manner. Based on the $LC_{50}$, dose the phenolic extracts were found to be the most toxic. Apart from the lethal effect, we are also reporting the adult emergence inhibiting effect of sublethal concentrations of the alkaloid, phenolic and terpenoid extracts in the treated larvae. Earlier *Z. jujuba* has been reported to be effective against *Culex pipiens* larvae in which the petroleum ether extract and oil had caused pathological effect on pupa and adult [10,11]. Toxicity and adult emergence inhibition activity has been reported in extracts from other plants also from different families against different species of mosquitoes [1–3,5,12,13]. But to avoid development of resistance in mosquito vectors, search and use of new environment friendly plant product is essential.

The effect of insecticides depend on the dose to which the insects are exposed. In a field population, the dose of the insecticide applied would vary over time and space, the age and health of the insects and other biotic and abiotic factors to which the insects are exposed [14]. If sublethal concentrations of any insecticide are effective for managing the vector population, its use can help in minimizing insecticidal dose. But apart from the killing effect, sublethal doses of insecticides may cause hormesis and pest resurgence [15]. Therefore the study of the effect of sublethal doses of a potential insecticide is important for efficient vector control strategies. In our study, exposure to sublethal concentration of the phenolic extracts from *Z. jujuba* leaf significantly prolonged the larval duration and reduced the adult lifespan of the treated mosquitoes. It is well established that moulting and metamorphosis in insects is mediated by ecdysteroids and juvenile hormones [16,17]. The disruption of normal life cycle of mosquitoes in the present study suggests a possible disturbance on the neuroendocrine cascade of the treated mosquitoes. Plant extract induced changes in growth period and longevity of insects have been reported by many workers [12,18–20]. Delay in the larval period might make the larvae vulnerable to life threatening problems such as attack of larval predators and pathogens, diseases, lack of proper breeding conditions, *etc.* Reduced life span of the adult results in lower reproductive success thereby decreasing the size of the population [21].

The results further showed that, the phenolic fraction reduced the fecundity and fertility of the treated insects and all the changes were significant in both the parent and F1 generation. Therefore, we conclude that, the phenolic extracts affect the reproductive health of the treated mosquitoes as well as the general fitness of the offsprings. Optimum fertility and production of large number of offsprings are two major factors on which the survival of an insect population depends. The decrease in fertility and fecundity of mosquitoes treated with phenolic extracts of *Z. jujuba* would adversely affect the reproductive capability and survivorship of the successive generations of the treated mosquitoes. Muthukrishnan and Pushpalatha [22] also reported decrease in fecundity and fertility of *Culex*, *Anopheles* and *Aedes* mosquitoes treated with extracts from *Calophyllum inophyllum*, *Rhinacanthus nasutus*, *Solanum suratense* and *Samadera indica*. Similar decrease in fecundity and hatchability was also reported in *Anopheles* larvae treated with alkaloids isolated from *Anona squamosa* seeds [23] and *Ae. aegypti* and *Aedes albopictus* larvae treated with *Ipomoea carica* extract [24].

Our study on the carbohydrate, lipid and protein level of the treated insects revealed that the effect on growth and development was accompanied by changes in the macromolecular levels in the treated mosquitoes of both parent and F1 generations. The appropriate accumulation of these nutrients during larval development has been shown to regulate juvenile hormone synthesis for activation of reproductive maturation. This influences the fertility and fecundity in the insects thereby affecting the survival of the species [25,26]. The total protein level significantly increased in the treated insects in both parent and F1 generation. In the parent generation, protein level increased 2.4 times in larvae and 1.3 times in pupae after treatment with the phenolic extracts. The increase may be due to the production of detoxifying enzymes or defense proteins. It is reported that, animal under toxic stress activates a compensatory mechanism, increases whole body protein content or may synthesize set of conserved polypeptides, collectively referred to as heat shock proteins [27,28]. Earlier the synthetic pyrethroid, deltamethrin and the insect neurotoxin, imidacloprid were reported to the increase the protein content of *Nilaparvata lugens* [29]. But in the F1 generation, although the protein levels of treated insects were significantly higher than control insects, they displayed a significant reduction of 1.30 folds in larvae and 1.03 folds in pupae as compared to the treated parents. This decrease may be either due to the reduction in the stress level of the treated insects or due to effect of the plant extracts on protein synthesis [3].

In contrast to protein level, the lipid, glucose and glycogen level decreased significantly in both larvae and pupae after treatment with the three extracts. Plant secondary metabolites may induce physiological stress in the treated insects leading to increase in energy metabolism to counteract the insecticide induced stress [2,30]. In the parent generation, glucose levels decreased 1.60 and 1.04 times in the larvae and pupae respectively after treatment with phenolic extracts. The glucose levels further decreased significantly in the F1 generation and were 1.8 and 1.3 times lower than that of larvae and pupae respectively in comparison to that of the parent generation. In case of glycogen too, the levels decreased 1.6 and 1.3 times in larvae and pupae of the parent generation and 1.4 times in both larvae and pupae of F1 generation. Glucose and glycogen are the predominant sources of energy reserve in insects and serves as an energy source to the post feeding larval and pupal stages [15]. Therefore decrease in the reserved energy level would be detrimental to normal morphology and metabolism of the treated insects. Kissoum and Soltani [31] also reported decreased glycogen level in *Drosophila* treated with growth inhibitor,

Spiromesifen in response to chemical stress. Further, lipid levels also decreased 1.4 and 1.14 times in larvae and pupae of parent generation and were 1.06 and 1.22 times lower than parent in case of larvae and pupae of F1 generation. The decrease in lipid level might be due to the shift in energy metabolism to lipid catabolism after depletion of carbohydrate reserves. Decrease in glucose and lipid was also reported in *Anopheles* and *Culex* larvae after treatment with extracts from antimalarial plant *Artemisia* [2]. Increased reduction in the glucose, glycogen and lipid levels in the F1 generation suggest presence of physiological stress due to the exposure to treatment in the previous generation followed by the insufficient accumulation of nutrients in the treated insects.

Thus, the effects on biochemical parameters revealed that the *Aedes* mosquitoes respond to toxicological stress caused by phenolic extracts from *Z. jujuba* through physiological changes. Similar physiological changes also observed in the successive generation (F1) suggests a continuous stress in reserved energy sources which would adversely affect the general fitness of the succeeding generations of the treated insects. From the present study we recommend the phenolic extracts of the leaves of *Z. jujuba* as potential candidate for the control of *Aedes* mosquitoes.

## Conflict of interest statement

We declare that we have no conflict of interests.

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