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Efficacy of Neem (*Azadirachta indica* A. Juss) Seed Kernel Extract against Dengue Vector, *Aedes aegypti* (Diptera: Culicidae)

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Laboratory bioassays were conducted to evaluate the larvicidal, ovicidal and repellent activity of neem (*Azadirachta indica* A. Juss) seed kernel extract (NSKE) on *Aedes aegypti*. The neem seed kernel powder was sequentially extracted with ethyl acetate, acetone, ethanol and distilled water. The extract exhibited dose dependent activity and produced significant mortality. The 24 h LC50 concentration of the extract was observed at, 11.45, 19.95 and 24.67 mg/l respectively. Mean per cent hatchability of the ovicidal activity was observed 120.00 h after treatment. The per cent hatchability was inversely proportional to the concentration of extract and directly proportional to the eggs. The crude extract of neem seed kernel shows significant repellency against *A. aegypti*. These results clearly reveal that NSKE served as a potential larvicidal, ovicidal and repellent agent against dengue vector mosquito, *A. aegypti*.

Key words: *Azadirachta indica, Aedes aegypti,* Larvicidal activity, Ovicidal activity, Repellent activity.

In recent years, vector-borne diseases (VBD) have emerged as a serious public health problem in countries of the South-East Asia Region, including India. India is endemic for six major vector-borne diseases (VBD) namely malaria, dengue, chikungunya, filariasis, Japanese encephalitis and visceral leishmaniasis. Dengue, dengue haemorrhagic fever and chikungunya are transmitted by *Aedes aegypti*. The symptoms of the disease are severe pain in the joints and muscles, skin eruptions. However, dengue fever is rarely fatal. The species breed, profusely in rainwater storage containers like cisterns, barrels, pots, etc. Dengue outbreaks are often associated with urban areas due to irregular potable water supply. Dengue fever continues in recurrent epidemic afflicting millions and causing thousands of deaths annually which is transmitted by *Aedes aegypti*. Mosquito are a serious threat to public health through which several dangerous diseases are transmitted in both animals and human beings[1].

Extensive use of chemical insecticides for control of vector borne diseases has created problems related to physiological resistance to vectors, adverse environmental effects, high operational cost and community acceptance[2]. Numerous plant products or phytochemicals have been reported either as insecticides for killing larvae or adult mosquitoes or as repellents for mosquito biting and are one of the best alternatives for mosquito control [2, 3].

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Neem tree, (*Azadirachta indica*) native of India, belonging to family Meliaceae is a fast growing evergreen tree ranging in height from 12–24 m. They are widespread in tropical and subtropical regions of the world, including semi-arid and wet- tropical regions[4]. Neem seeds contain approximately 99 biologically active compounds of which azadirachtin, nimbin, nimbidin and nimbolides are major molecules. Many of these derived products have antifeedant, ovicidal activity, fecundity suppression besides insect growth regulation and repellency against insects[5-10]. Azadirachtin, a biologically active compound has been promoted as a new insecticide that is considered more eco- friendly than synthetic insecticides. The pesticidal efficacy, environmental safety and public acceptability of neem and its products for control of crop pests has led to its adoption into various mosquito control programmes[8, 11].

The problems associated with synthetic insecticides necessitated investigations in to phytochemicals for mosquito control, since they are environmental friendly, biodegradable, target specific, and locally available in mosquito endemic area[12]. Some phytochemicals act as toxicant (insecticide/larvicide) both against adult as well as larval stages of mosquitoes. While other interfere with growth and growth inhibitor or with reproduction or produce an olfactory stimuli thus acting as repellent or attractant[5]. Phytochemicals with diverse mode of actions may be effective against resistant vector species and can be easily integrated with other mosquito control measures in both private and public mosquito control programmes[13].

This study investigates the effects of neem seed kernel extracts against larvicidal, ovicidal and repellent activities of dengue vector, *Aedes aegypti* in the laboratory.

MATERIAL AND METHODS

Collection of Plant Material and Preparation of Extract

Neem seeds were collected from mature disease-free trees in and around A.M.U. Campus, Aligarh (U.P.) India. The plant material was identified and confirmed by the taxonomists, Department of Botany, A.M.U. Aligarh, (U.P). The dried neem seeds were decorticated to remove the kernels and air dried for 5 days before pulverization with an electric blender and sieved with 40 mesh screen to obtain a fine powder. The neem seed kernel powder (1.0 kg) was then subjected to extraction in various solvents viz, ethyl acetate**,** acetone and ethanol, using Soxhlet apparatus. The extract was filtered through Whatman number one filter paper and the filtrates were evaporated to dryness in a water bath. The residue was then made in to a 1% stock solution with distilled water. From this stock solution, various test concentrations were prepared, which were used for the bioassays.

Insect Culture

The dengue vector mosquito, *A.aegypti* was reared in the laboratory. The larvae were fed on dog biscuits and yeast power in the 3:1 ratio. The adults of both sexes were fed with 10 % glucose solution. In addition, females were fed on blood meal twice a week from restrain chicken with shaved abdominal feathers. Mosquitoes were held at 28 ±2ºC and 75-85% relative humidity (RH).

Larvicidal activity

The Larvicidal activity of NSKE was evaluated according to the method recommended by WHO[14]. Various concentrations of the test samples were prepared in 500 ml glass beakers containing 250 ml of distilled water. Third instar larvae of *A.aegypti* were exposed to each of the test solution as well as control. Six replicates were maintained at a time for each experiment. LC_{50} was calculated after 24 hrs by probit analysis[15].

Ovicidal activity

The ovicidal activity of NSKE was evaluated according to the method performed by Su and Mulla[11]. Preliminary tests indicated that ovicidal activity of NSKE was influenced by the eggs and concentration of extract. To study this relationship, 100 gravids of *A.aegypti* were placed in a screen cage, where 9 oviposition cups were introduced for oviposition 30 min before the start of the dusk period. Of these 9 cups, 8 were each filled with test solution of 20, 40, 60, 80, 100, 120, 140 and 160 mg/l and one was filled with 100 ml of solvent containing water that served as a control. The eggs laid (most of the eggs were laid within 4 hrs) in different concentration of leaf extract were collected immediately after oviposist and used in eggs age test after 12-18 h interval after oviposition respectively. Eggs were selected at random and

individually transferred to the different concentration of extract for 3 h. After treatment the eggs from each concentration were individually transferred to distilled water cups for hatching assessment after counting the eggs under microscope. The test was replicated six times. The hatch rate was assessed 120 h post treatment by the following formula:

$$\frac{\text{Number of hatched larvae}}{\text{Total number of eggs}} \times 100$$

Repellent activity

The minutes of protection in relation to dose method was used to evaluate repellent activity of NSKE. Three day old blood starved female *Aedes aegypti* mosquitoes (100) were kept in net cages (45 cm × 30 cm × 45 cm). The arms of the test person were cleaned with distilled water. Following air drying, 25 cm^2 area of the arm (dorsal side) was exposed to the mosquitoes while the remaining portion of the arm was covered with rubber gloves. The crude extract was dissolved in ethanol. The concentration of crude extract at 2, 4 and 6 mg/cm^2 was applied. The control and treated arms were introduced simultaneously into the cages. The time of the test dependent on whether the target mosquitoes day-or night biters. *Aedes aegypti* was tested during the day time from 07:00 to 17:00 h. The number of bites was counted over 3 min in every 30 min intervals (it is make out 30 min protection). The experiment was conducted six times. All the experiments were conducted at a temperature of 28 ± 2°C and relative humidity of 80 ± 2%. The percentage of protection was calculated by using the following formula.

(No. of bites received by control area) - (No. of bites received by treatment area) / (No. of bites received by control area) ×100

Statistical Analysis

Statistical evaluation was done using Statistical Package of Social Sciences (SPSS). Level of significance was set at $P < 0.05$.

Table 1. Larvicidal action of neem seed kernel extract (NSKE) against *Aedes aegypti*

Solvent	Concentration (mg/l)	Larval Mortality (%)	LC50 (mg/l)	95% confidence limit (mg/l)		LC90 (mg/l)	Chi-square
				LCL	UCL		
Ethyl acetate	25	97.00	11.45	9.93	14.13	22.48	10.361[a]
	20	87.66					
	15	73.00					
	10	48.33					
	05	32.66					
	Control	3.66					
Acetone	50	99.00	19.95	15.83	24.45	38.65	15.282[a]
	40	92.33					
	30	77.66					
	20	54.00					
	10	37.33					
	Control	3.66					
Ethanol	50	95.00	24.67	17.76	33.74	48. 37	21.461
	40	77.33					
	30	56.00					
	20	42.33					
	10	35.33					
	Control	3.66					

[a]Significant at $p < 0.05$.

RESULTS

The toxicities of the crude extract from neem seed kernel extract (NSKE) to third instar *A.aegypti* larvae was noted, and the LC50, LC90, 95% confidence limits of LCL and UCL and *chi*-square were also calculated (Table 1). The ethyl acetate extract was found to be more effective than the other extract against larvae of *A.aegypti* with LC50 value of 11.45 mg/l. The *chi*-square values were significant at $P<0.05$ level. In laboratory test, the oviposition cups treated with different concentration of NSKE in 100 ml of distilled water received different number of eggs at different concentrations. The solvent containing water served as a control received only small amount of eggs. The different age of eggs *A.aegypti* treated with different concentrations of NSKE caused ovicidal resulting in failure to hatch the eggs (Table II). The crude extract of neem seed kernel shows significant repellency against *A.aegypti* (Table III). It shows that repellency depends on the concentration of crude extract.

Table 2. Effect of neem seed kernel extract (NSKE) on the hatchability of the eggs of *Aedes aegypti*

Egg Hatchability (%)									
		Concentration of extract (mg/l)							
Solvent	Control	20	40	60	80	100	120	140	160
Ethyl acetate	100.00	84.66	70.33	56.33	33.66	16.33	NH	NH	NH
Acetone	100.00	89.33	83.66	64.33	43.66	27.66	14.33	NH	NH
Ethanol	100.00	95.66	86.33	71.66	52.33	38.66	28.66	15.33	NH

NH- No Hatchability (100% mortality)

Table 3. Repellent activity of neem seed kernel extract (NSKE) against vector mosquito *Aedes aegypti*

Solvent	Concentration of leaf extract (mg/cm²)	Mean number of bites received in control	Mean number of bites received in treated	Mean number of hrs of 100% protection	Total % of protection for 10 hrs
Ethyl acetate	2.0	50.0 ± 1.6	27.0 ± 1.4	3.00	49.00
	4.0	50.0 ± 1.2	21.0 ± 0.6	5.15	60.00
	6.0	51.0 ± 1.4	18.0 ± 1.2	6.45	70.33
Acetone	2.0	51.0 ± 0.6	31.0 ± 1.6	2.30	44.66
	4.5	51.0 ± 1.2	25.0 ± 1.2	4.30	56.79
	6.0	50.0 ± 0.2	20.0 ± 1.4	5.45	65.33
Ethanol	2.0	51.0 ± 1.2	38.0 ± 0.6	2.15	33.69
	4.5	51.0 ± 0.8	34.0 ± 0.6	3.30	42.43
	5.0	50.0 ± 1.6	27.0 ± 1.2	5.15	50.79

Values are mean of six replication ± SD

DISCUSSION

The larvicidal, ovicidal and repellent activities of neem seed kernel extracts against *A. aegypti* were studied. The findings of this report will serve as base line data for mosquito control in India. The neem seed extracted with ethyl acetate, ethanol, hexane, pentane, acetone, esters and dichloromethane as well as mixture of any of these solvents with water possessed insecticidal activities[6] Simple crude extracts from plants have been used as insecticides in many countries for

centuries[16]. Crude plant extracts often consist of complex mixtures of active compounds. Advances of using complete mixture may act synergistically[17], they may show greater overall bioactivity compared to the individual constituents[18]. The mosquito larvicidal properties of the leaf extract of a herbaceous plant *Ocimum canum* against *Aedes aegypti*. The LC50 values for 2nd, 3rd and 4th larvae were 177.82, 229.08 and 331.13 ppm respectively[19]. The egg rafts aged for 0, 4, 8, 12 and 24 hr were exposed to 10 ppm neem suspensions for 36 hrs and the ovicidal activity was only attained in the egg rafts deposited directly (0 hr old) in neem suspensions, not in those with ages of 4-24 hr. In this study, the exposure period also played a crucial role in causing toxicity[11]. Methanolic leaf extract of *Cassia fistula* was tested for larvicidal and ovicidal activity against *Culex quinquefasciatus* and *Anopheles stephensi*. The extract was found to be more lethal to the egg and larvae [20]. Repellent activity of *Ferronia elephantum* (Rutaceae) leaf extract against *Aedes aegypti* has been demonstrated previously, where in the total percentage of protection of *Ferronia elephantum* was estimated to be 45.8% of 1.0 mg/cm^2 and 59.0% at 2.5 mg/cm^2 for 10h [21]. Isolation of repellent ingredients from *Lantana camera* (Verbenaceae) flowers and their repellency against *Aedes* mosquitoes has also been demonstrated. One application of this fraction gave 100% protection for 2 h and may protect 75.8% at 7 h [22]. The finding of the present investigation revealed that the NSKE possess remarkable larvicidal, ovicidal and repellent activity against medically important vector mosquitoes. The extract might be used directly as larvicidal and ovicidal agent in small volume aquatic habitats or breeding sites of limited size around human dwellings and also possess good repellent activity. Further investigations are needed in this direction to strengthen this hypothesis and explore the exact mechanism of NSKE action on *A. aegypti*.

CONCLUSIONS

These results clearly reveal that the crude extract of neem seed kernel served as a potential larvicidal, ovicidal and repellent agent against dengue vector mosquito, *Aedes aegypti*. This technique is environmental friendly, biodegradable, less expensive, and could be made available locally in mosquito endemic area. Potentials for adoption in mosquito management programmes cannot be overemphasized. Further research on the potentials of NSKE in field conditions necessitate practical implementations of mosquito control programmes to protect human populations from the scourge of mosquito-borne diseases in India and other mosquito endemics areas of the world.

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