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INTERNATIONAL JOURNAL OF INNOVATION RESEARCH

RESEARCH ARTICLE

International Journal of Innovation Research - Vol. 1, Issue, 1, pp.012 - 018, January, 2013

OVICIDAL AND ADULTICIDAL ACTIVITY OF *NERIUM OLEANDER* EXTRACT AGAINST *ANOPHELES STEPHENSI* LISTON (INSECTA: DIPTERA: CULICIDAE)

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A R T I C L E I N F O

Article History:

Received 10th December, 2012
Received in revised form 20th, December, 2012
Accepted 5th January, 2013
Published online 31th January, 2013

Key words:
Nerium oleander, Anopheles stephensi, Malaria, Ovicidal activity, Adulticidal activity, Smoke toxicity.

A B S T R A C T

Objective: To test the smoke toxicity effect of *Nerium oleander* on biting activity and ensured population of *Anopheles stephensi* and to prepare different solvent extracts of *Nerium oleander* to determine it's ovicidal and adulticidal efficacy against *Anopheles stephensi.*

Methods: In the present study, *Nerium oleander* leaves, stem and root were collected, washed and shade dried in enamel trays at room temperature. Then the dried plant parts were powdered with an electric blender. From the powder 200g of the plant material were extracted with 2.5 litres of different solvents such as aqueous solution, ethyl acetate and ethanol for 8 hrs in a soxhlet apparatus. The crude plant extracts were evaporated to dryness in rotary vacuum evaporator. One gram of the plant residue was dissolved in 100 ml of acetone (stock solution), considered as 1% stock solution. From this stock solution concentrations were prepared ranging from 100 to 300 ppm. The extract was tested for its ovicidal and adulticidal activity, and mosquito coil was prepared using different plant parts and its smoke toxicity was noted against the malaria vector under laboratory conditions.

Results: Ovicidal and adulticidal activity was measured by preparing different concentrations of different solvent extracts of N.oleander. Smoke toxicity effect of the plant by preparing Mosquito coil from different parts of *N.oleander* powder. The mortality rates of crude using solvent extract at 300ppm were higher than all other concentrations when tested against the adult mosquitoes at 24h-48h of exposure. Smoke exposed females have produced fewer eggs when compared to the non-exposed female *An.stephensi* mosquitoes.

Conclusions: The results indicate promising ovicidal and adulticidal activity against *Anopheles stephensi.* The plant parts and solvent used for extraction, phototoxic activity and the geographical origin of the plant compound are important factors in the efficacy of a phytochemical and the results showed that the mosquitocidal mortality was dose dependent. The use of plant *N. oleander* as a biological insecticide is a rapid, environmentally safer, and greener approach for mosquito control.

INTRODUCTION

Mosquito serve as crucial vectors for a number of arboviruses (arthropod-borne viruses) and parasites that are maintained in nature through biological transmission between susceptible vertebrate hosts by blood feeding on hosts, they are responsible for malaria, encephalitis, dengue, rift valley fever, yellow fever and other vector-borne diseases. WHO has declared the mosquitoes as "public enemy number one" [1]. Malaria affects more than 600 million people and causes several million deaths every year in tropical countries [2]. *Anopheles stephensi* is a major malaria vector in India. With an annual incidence of 300–500 million clinically manifest cases and a death toll of 1.1–2.7 million, malaria is still one of the most important communicable diseases. Mosquito control is the key measure to prevent spreading these diseases. Synthetic insecticides have been widely developed and are extensively used because of their effectiveness and easy storage. However, their extensive use has brought some severe side effects, like environmental disturbances, pest recovery, pest resistance, lethal effects on non-target organisms, and toxicity to users and consumers [3]. Evaluating and using botanical pesticides, either as crude or formulated extracts, is an alternative strategy.

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One of the most effective alternative approaches under the biological control programme is to explore the floral biodiversity and enter the field by using safer insecticides of botanical origin as a simple and sustainable method of mosquito control. The use of conventional insecticides has raised some concern about their threat to the environment and development of insecticide resistance in insects [4], there is an imperative need for the development of safer, alternative crop protectants such as botanical insecticides and repellents. Crude plant extracts often consist of complex mixtures of active compounds, they may show greater overall bioactivity compared to the individual constituents [5]. The deleterious effects of crude plant extracts on insects are manifested in several ways, including toxicity [6], feeding inhibition [7]. To date, a number of phytochemicals with biological activity against immature and adult mosquitoes have been described [8-9]. The fecundity and egg hatchability were also subsequently affected due to the impact on larvae.

Biological control may involve the use of plant, animal, or fungal species or components to reduce the survival, growth, or reproduction of the mosquito species. Repellents are used as personal protection methods against biting arthropods with the major aim of avoiding nuisance. Insect repellents are considered useful alternatives where other control measures are neither practical nor possible. Pyrethrin-based mosquito coils are widely used in most of the countries which protects the people from mosquito bites through their repellent effects, knocking-down and killing effects. Synthetic pyethroids such as D-allethrin and D-*trans* allethrin possessing potent insecticidal activity with low mammalian toxicity have increasingly been used as substitute for natural pyethrins in many mosquito coil formulations. However, prolonged exposure to these chemicals may lead to local irritation, severe allergic dermatitis and systemic allergic reactions. It may include nausea, vomiting, tinnitus, headache, and other CNS disturbances [10]. The most common mosquito repellent formulation available on the market contain DEET (N, N-diethyl-m-toluamide), which has shown excellent repellence against mosquitoes and other biting insects [11]. However, side effects after the application of DEET vary from mild to severe, it has an unpleasant smell, oily feel and high skin penetration, and it can dissolve plastic and synthetic rubber [12]. These effects highlight the urgent need for development of new, effective, safe, and eco-friendly repellents. Plant-based mosquito repellents are a viable source of material for use in protection against mosquitoes and mosquito-transmitted diseases [13] and have some advantages over the current gold-standard synthetic repellent, N, N-diethyl-m-toluamide (DEET) [14]. The phytochemicals derived from plant sources can act as larvicides, insect growth regulators, repellents and ovipositional attractants and have deterrent activities observed by many researchers [15].

In this study, we determined the biological activity of different solvent leaf extracts of *Nerium oleander* on ovicidal, adulticidal, blood feeding acivity of female *Anopheles stephensi* and also tested the effect of smoke on *An.stephensi*.

MATERIALS AND METHOD

Collection of Plant

Fresh leaves of *N. oleander* (Fig 1) were collected from the campus of Bharathiar University, India. The plants were identified by a taxonomist from the Department of Botany, Bharathiar University,Coimbatore. The voucher specimen was numbered and kept in Entomology ResearchLaboratory for further reference.

Preparation of Plant Extracts

N. oleander leaves were washed with tap water and shade dried at room temperature. The dried plant materials were powdered by an electrical blender. From the powder, 500g of the plant material was extracted with 1.5L of different solvent (ethyl acetate, aqueous solution and ethanol) for a period of 8 hrs in a soxhlet apparatus [16]. The crude plant extracts were concentrated at reduced temperature on a rotary evaporator and stored at a temperature. One gram of the plant residue was dissolved in 100 ml of acetone (stock solution) considered as 1% stock solution. From this stock solution concentrations were prepared and these solutions were used for ovicidal and adulticidal bioassays.

Mosquito collection and rearing

The eggs of *Anopheles stephensi* were collected from the National Centre for Disease Control field station of Mettupalayam, TamilNadu, India. These eggs were then brought to the laboratory and transferred to 18-cm L×13-cm W×4-cm H size enamel trays containing 500mL of water where they were allowed to hatch. Mosquito larvae were reared (and adult mosquitoes held) at 27 °C±2 °C and 75–85 % RH in a 14:10 (light/day) photoperiod. The eggs and larvae obtained from the above said stock were maintained for many generations in the laboratory and were used for further experiments.

Maintenance of Pupae and Adult

The pupae were collected from culture trays and were transferred to glass beakers containing 500 ml of water with the help of a sucker. The glass beaker containing pupae was then kept in 90 x 90 x 90 cm size mosquito cage for adult emergence. The cage was made up of wooden frames and covered with polythene sheets on four side (two laterals, one back and other one upper) and the front part was covered with a muslin cloth. The bottom of the cage was fitted with strong cardboard. The freshly emerged adults were maintained at 27.2°C, 75% - 85% RH, under 14L: 10D photoperiod cycles. The adults were fed with 10% sugar solution for a period of three days before an animal blood meal was provided.

Blood feeding of adult Anopheles stephensi and egg laying

The females were fed by hand every alternate day at 6.00 P.M. Feeding mosquitoes on human arm for experimental purposes [17-18]. Both females and males were provided with 10% glucose solution [19] on cotton wicks. The cotton was always kept moist with the solution and changed every day. Glucose as well as ordinary sugar appeared equally attractive to the mosquitoes [20]. An egg trap (cup) lined with filter paper containing pure water was

always placed at a corner of the cage. This arrangement made collection of eggs easier.

Ovicidal activity assay

The method of Su & Mulla [21] was followed to test the ovicidal activity. The leaf extract was diluted in the dfferent solvents (aqueous solution, ethyl acetate and ethanol) to achieve different concentrations. The freshly laid eggs were collected by providing ovitraps in mosquito cages. Ovitraps were kept in the cages 2 days after the female mosquitoes were given a blood meal. The eggs were laid on filter paper lining provided in the ovitrap. After scoring, 100 gravids were placed in a screen cage where ten oviposition cups were introduced for oviposition 30 min before the start of the dusk period. One hundred freshly laid eggs of *An. stephensi* were exposed to each concentration of different solvent extract of *N.oleander* until they hatched or died. Each concentration was replicated five times. Eggs exposed to acetone in water served as control. After the treatment, the eggs from each concentration were transferred to distilled water in a cup and counted under a microscope for hatching assessment. The hatch rate was assessed after 48 h post treatment [22].

Adulticidal activity

Five to ten day old sugar-fed adult female mosquitoes were used for this assay. The *N.oleander* leaf extracts were diluted with different solvents (aqueous solution, ethyl acetate and ethanol) to make different concentrations of each solvent. The diluted plant extracts were impregnated on filter papers (140 ×120 mm). A blank paper consisting of only ethanol was used as control. The papers were left to dry at room temperature to evaporate off the ethanol overnight. Impregnated papers were prepared fresh prior to testing. The bioassay was conducted in an experimental kit consisting of two cylindrical plastic tubes both measuring (125 ×44 mm) following the WHO method [23]. One tube served to expose the mosquitoes to the plant extracts and another tube was used to hold the mosquitoes before and after the exposure periods. The impregnated papers were rolled and placed in the exposure tube. Each tube was closed at one end with a 16 mesh size wire screen. Sucrose-fed and blood starved mosquitoes were released into the tube, and the mortality effects of the extracts were observed every 10 min for 3 h exposure period. At the end of 1, 2, and 3 h exposure periods, the mosquitoes were placed in the holding tube. A Cotton pad soaked in 10% sugar solution with vitamin B complex was placed in the tube during the holding period of 24 h. Mortality of the mosquitoes was recorded after 24-48h.

Smoke Toxicity Test

N. oleander parts were used for smoke toxicity assay. The mosquito coil was prepared by following method of Saini [24] with minor modifications by using 4 grams of plant powdered sample considered as active ingredient, two grams of saw-dust as binding material and two grams of coconut shell charcoal powder as burning material. All the three were thoroughly mixed with distilled water to form a semisolid paste. Mosquito coils (0.6 cm thickness) were prepared manually from the semisolid paste and were shade dried. The control coils were prepared without plant ingredient. The duration of the exposure was 6 hours per coil.

The experiments were conducted in glass chamber measuring 140 x 120 x 60 cm. A window measuring 60 x 30 cm was situated at mid bottom of one side of the chamber. Three or four day's old blood starved hundred adult female mosquitoes, fed with sucrose solution, were released into the chamber. A belly shaven pigeon was kept tied inside the case in immobilized condition. The experimental chamber was tightly closed. The experiment was repeated five times on five separate days including control using mosquitoes of same age groups. The data were pooled and average values were subsequently used for calculations. Control was maintained in two sets. One set was run with coil lacking the active ingredient of plant powder (control 1) another one is Mortein coil which is used positive control to compare the effectiveness of plant coils. After the experiment was over the fed, unfed (active and dead) mosquitoes were counted. The protection given by the smoke from plant samples against the biting of *An. stephensi* was calculated in terms of percentage of unfed mosquitoes due to treatment.

$$\%\ Protection = \frac{No.of\ unfed\ mosquitoes\ in\ treatment - No.of\ unfed\ mosquitoes\ in\ control1}{No.of\ mosquitoes\ treated} \times 100$$

The alive mosquitoes fed with blood meal were reared in mosquito cage, measuring 30 x 30 x 15 cm. The top and bottom of the cage were fit with glass and all other sides were covered with muslin cloth. Water soaked rasins and 5% sucrose solution soaked in cotton balls were kept as food inside the cage. Water containing powdered yeast and dog biscuits were also kept inside the cage in a glass bowl to collect eggs. The eggs from the cage were collected daily till all the mosquitoes died. 50 to 100 eggs were allowed to hatch in each plastic tray measuring 30 x 25 x 6 cm, containing about 2.5 litres of unchlorinated tap water. The larvae hatched from the eggs were fed with a mixture of dog biscuits and yeast powder in the ratio of 2:1 and water in the tray was changed daily. The number of larvae hatched was counted at second instar stage. The reduction in the population from the smoke treated mosquitoes was calculated using the formula:

$$Population\ reduction\ (\%) = \frac{No.of\ larvae\ hatched\ in\ control\ 1 - No.of\ larvae\ hatched\ in\ treated}{No.of\ larvae\ hatched\ in\ control\ 1} \times 100$$

RESULTS

Ovicidal activity of N.oleander leaf extracts against An. stephensi eggs

The ovicidal activity with different solvent extracts of *N.oleander* leaf was observed against the *An. stephensi* eggs. A total number of 100 eggs were used in control as well as for the test, and the percentage of egg hatchability in control (acetone mixed with distilled water) was 100%. The eggs of *An. stephensi* treated with different solvent extracts with different concentrations of leaf extract were failed to hatch. And from the results obtained, it is well

Table 1 Ovicidal activity of different solvent extracts of *N.oleander* extracts against *An.stephensi.*

Solvent	% of egg hatchability in control	% of egg hatchability Concentration (ppm)				
		100	150	200	250	300
Ethyl acetate	100± 0.00	77.6±2.10	74.9±1.38	61.0±2.80	41.50±2.38	29.90±2.45
Aqueous solution	100 ±0.00	62.40±2.0	50.00±3.66	35.70±2.4	21.50±2.17	NH
Ethanol	100± 0.00	60.30±2.98	47.30±2.44	39.80±2.60	NH	NH

NH : No-hatchability (100% mortality)

Table 2 Adulticidal activity of different solvent extracts of *N.oleander* against *An.stephensi.*

Name of the extract	Concentration (ppm)	% Mortality ± SD	LC_{50} (LFL–UFL)	LC_{90} (LFL–UFL)	χ^2
Ethyl acetate	Control	0.0±0.0	164.613 (145.693-180.400)	335.161 (305.020-381.210)	2.144*
	100	32.30±2.67			
	150	47.40±2.46			
	200	58.00±3.22			
	250	70.00±2.18			
	300	88.00±1.59			
Aqueous solution	Control	0.0±0.0	146.124 (131.401-158.466)	265.120 (247.705-288.789)	4.175*
	100	35.60±2.38			
	150	48.30±2.83			
	200	68.50±2.64			
	250	85.20±2.39			
	300	98.10±1.79			
Ethanol	Control	0.0±0.0	125.491 (49.573-159.491)	235.412 (197.590-340.517)	9.757*
	100	43.70±3.62			
	150	59.80±2.45			
	200	71.50±2.33			
	250	95.00±2.45			
	300	100±1.03			

LFL lower fiducidal limits, *UFL* upper fiducidal limits, χ^2 Chi square value, *Significant at P< 0.05 level

Table 3 Smoke toxicity effect of *N. oleander* parts against biting activity of *An. stephensi.*

N. oleander Parts used	No. of mosquitoes used	Fed mosquitoes	Unfed mosquitoes		Total No. of unfed mosquitoes	% unfed over control I
			Alive	Dead		
Leaf	100	12	40	48	88	59
Stem	100	31	33	35	69	40
Root	100	18	40	42	82	53
Control I*	100	71	29	0	29	0
Control II**	100	14	33	61	94	65

Control I* =Negative control - coil without plant material
Control II** = Positive control - mortein coil

Table 4 Smoke toxicity effect of *N. oleander* parts against ensured population of *An. Stephensi*

N. oleander parts used	No. of mosquitoes used	No. of egg rafts laid by fed mosquitoes	Total No. of eggs	Total No. of larva hatched from the egg rafts	% of reduction in population over control I
Leaf	25	4	436	218	74.85
Stem	25	7	748	525	39.44
Root	25	10	980	590	31.94
Control I*	25	14	1075	867	0
Control II**	25	4	456	220	74.62

Control I* = Negative control – coil without plant material
Control II** = Positive control – mortein coil

understood that the percentage of egg hatchability was decreased with the increasing concentration of the extract. Ovicidal activity with different solvent leaf extract of *N.oleander* against *An.stephensi* at 100,150,200,250 and 300ppm were noted in Table 1. With each extract at a concentration of 100 ppm, the percentage of hatchability was very high and nil hatchability was recorded when the concentration of extract was increased to 300 ppm in the case of aqueous and ethanol extract.That is, there was an increase in egg mortality with increase in *N.oleander* leaf extract concentration, which was highly dose-dependent.

Figure 1 *Nerium oleander*

Adulticidal activity of N.oleander leaf extracts against An. Stephensi

Adulticidal activity of *N.oleander* leaf extract against adult *An. stephensi* is presented in Table 2. The three solvent extracts used for the study showed a high range of adulticidal activity against the malaria vector. Among the three extracts, ethanol extract of *N.oleander* has highest adulticidal activity followed by aqueous extract and ethyl acetate extract respectively. Highest adulticidal activity was observed with higher concentration of the plant leaf extract. With each concentration, the adult malaria vector has some restless movement, delayed egg laying and it also refuses to feed blood meal for a few period of time and died. The activity of the vector was also dose-dependent.

Smoke toxicity effect of N.oleander against An.stephensi

Table 3 provides the results of smoke toxicity effect of *N. oleander* plant against the malaria vector, *An. stephensi*. Of the three plant parts used (leaf,stem and root), higher mortality was noted with the smoke emerged from the leaf made coil than other two parts. Among the 100 *An.stephensi*, there were 12 fed and 88 unfed mosquitoes counted after the treatment with *N. oleander* leaf made coil. The comparison of positive control with the plant made coil has showed good smoke toxicity effect on *An. stephensi*.

Table 4, provides the results of population reduction effect of *N. oleander* (leaves, stem and root) smoke on ensured population of malaria vector, *An. stephensi*. In this case also *N.oleander* leaves have showed much more effectiveness than other parts of the plant. After the treatment with *N.oleander* leaf smoke, 25 mosquitoes laid 436 eggs, in which only 218 eggs were hatched. The percentage of reduction was 74.85. In the positive control, only 456 eggs were laid and the total numbers of eggs hatches were 220 and the percentage of reduction was 74.62. The *N. oleander* plant leaf and stem smoke exhibited higher efficacy of percentage reduction on ensured population of *An. stephensi* than the root of the *N.oleander*.

In the present study the smoke emerged from *N.oleander* considerably affected the adult mosquito survival and pronounced high mortality against malaria vector. And the treated individual mosquitoes laid minimum number of eggs. Hence, this plant extract can be used for the control of *An. stephensi.*

DISCUSSION

N.oleander with different solvent leaf extracts exerted ovicidal, adulticidal and smoke toxicity effect against the malaria vector, *An. stephensi*. The leaf extract treated eggs exhibited a delayed hatchability and this may be due to the action of phytochemicals present in the extract. The larvae which hatched out of the treated eggs were dead within few hours. In the case of ovicidal activity, exposure to freshly laid eggs was more effective than to the older eggs. A study reported that the younger age groups of egg rafts or eggs showed poor hatchability rate when exposed to higher concentrations of extract and older age groups of egg rafts or eggs showed high hatchability rate when exposed to lower concentrations of extract [25]. The methanol containing water that served as a control showed 94% hatchability in 0–3-h-old egg rafts/eggs, but the 100% hatchability was noted in egg rafts/eggs beyond the age of 0–3 h old in leaf methanol (90%) extract of *Cassia fistula* against egg raft of *Culex quinquefasciatus* [26]. The bioactive compound Azadirachtin isolated from *Azadirachta indica* showed complete ovicidal activity in eggs of *Culex tarsalis* and *Culex quinquefasciatus* exposed to 10 ppm concentration [27].

The active substances of *N.oleander* were toxic to the malaria vector and it may have the capability to interfere with the endocrine system, neuronal system and reproductive system of *An. stephensi* which may be the reason to show refusal to take the food even though it was in starved condition, restless movement, delayed egg laying and so on. Egg maturation in *Ae.aegypti* is a nutrient dependent process that is controlled by a regulated endocrine cascade involving several mosquito tissues, including the midgut, hemolymph, brain, ovaries, and fat body [28]. Female mosquito takes a blood meal is one of the major events of egg maturation process which includes the accumulation of egg yolk proteins, lipids, and iron by developing oocytes [29-31]. In *Ae.aegypti*, a key stage of egg development is vitellogenesis, the tissue-specific expression, synthesis, and secretion of the egg yolk protein precursors, such as vitellogenin (*Vg*), by the mosquito fat body [28]. Activation of vitellogenesis is dependent on both the presence of the steroid hormone 20-hydroxyecdysone, and amino acids derived from the digested blood meal [32].

The search for physiological mechanisms underlying egg development in mosquitoes has focused on blood-feeding species, largely due to their potential to act as disease vectors. Females of mosquito species that must ingest blood meals for the first and subsequent ovarian cycles are considered anautogenous. Soon after female *Ae.aegypti* feeds, the corpora allata (CA) begin secreting juvenile hormone (JH), which induces differentiation (competency) of ovaries and fat body. Once this pre-vitellogenic phase is complete, oogenesis is arrested until a blood meal is obtained. The act of feeding and presence of blood in the midgut initiate an endocrine cascade that ultimately results in egg maturation and oviposition. A steroidogenic gonadotropin released from the brain stimulates *Ae. aegypti* ovaries to secrete ecdysteroids [33]. In addition to endocrine regulation, products of blood meal digestion play a role in activating anautogenous oogenesis. For *Ae.aegypti* and several *Anopheles* species, the nutritional environment experienced by a female larva dictates her adult body size and resulting teneral reserves [34-35]. Teneral reserves affect important female reproductive processes, such as utilization of reserves, fecundity, longevity and blood meal consumption and utilization [34-37].

The active substances of *N.oleander* were toxic to the malaria vector, *An.stephensi*. Thus, these plant products can be an economically viable form of personal protection against mosquito vector. The smoke exposure affects the central nervous system and hence affects the neuroendocrine system to inhibit the hatachability of eggs and reduces the egg laying capacity as well the egg hatachability of the mosquitoes. Moreover, this kind of plant derived product does not cause any ill-effect to other beneficial organism [38].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are thankful to Dr. K. Sasikala, Professor and Head, Department of Zoology, Bharathiar University, Coimbatore, India for the facilities provided to undergo the study successfully.

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