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Research Article

LARVICIDAL, REPELLENT AND SMOKE TOXICITY EFFECT OF NEEM PRODUCTS AGAINST MALARIALVECTOR, *ANOPHELES STEPHENSI*

K. Murugan[1]*, G. James Pitchai[1], P. Madhiyazhagan[1], T. Nataraj[1], A. Nareshkumar[2], Jiang-Shiou Hwang[3], R. Chandrasekar[4], M. Nicoletti[5], A. Amsath[6] and Ranjeet Bhagooli[7]

[*1]Division of Entomology, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore-641 046, Tamil Nadu, India.

[2]Division of Entomology, Department of Zoology, Periyar University, Salem, India.

[3]Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan, 202.

[5]Department of Biochemistry and Molecular Biophysics, 238 Burt Hall, Kansas State University, Manhattan 66506, KS, USA.

[5]Department of Environmental Biology, University Sapienza of Rome, P.le A., Moro, 5-00185, Italy.

[6]PG and Research Department of Zoology, Khadir Mohideen College, Adirampattinam-614 701, Tamil Nadu, India.

[7]Department of Biosciences, Faculty of Science, University of Mauritius, Reduit- 80837, Mauritius.

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ABSTRACT

Vector control plays a key role in prevention and control of major vector-borne diseases and often constitutes the first line of activity in case of epidemics of vector-borne diseases, and particularly, malaria. Chemical control (use of pesticides) is still the most important element in the integrated approach to vector control. But they are non-selective and harmful to other beneficial organisms. Some of the insecticides are carcinogenic agents and are positively dangerous and even carried through the food chain which in turn affects the non-target organism. In view of the above, the uses of biologically-active plant materials with anti-mosquitocidal properties and ecofriendly-biopesticides are attracted in recent years, because of their biodegradable nature and being relatively safer to human and other non-target organism in the environment. The present paper is to investigate on the larvicidal, pupicidal, smoke repellency effect of neem products against malarial vector, *Anopheles stephensi*. Six neem limonoids (purity>99%), namely azadiractin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin were sent from Central Research Laboratories, Taiyo Kagaku Co Ltd., Japan. Larvicidal bioassays were conducted at the laboratory with neem limonoids the lethal concentrations ($LC_{50,}$ LC_{90}) were worked out by Abottts' formula. Repellency bioassay was done by human volunteers by using neem oil which was procured from the Local Neem Oil Mill, Kalverrampalayam, Coimbatore-641 046, India. Smoke toxicity was performed on the adult female mosquitoes at the laboratory by using neem seed kernel power and it was collected from the Bharathiar University Campus, Coimbaotore-641 046, India. The neem products also had significant larvicidal activity. The larval mortality was dose dependant. The LC_{50} and LC_{90}values of Azadirachtin treatment at 0.50, 1.0 and 1.5 ppm concentrations was 0.299% and 1.061%, respectively. After treatment of neem oil at 0.50, 1.0 and 1.5 ppm concentrations were 0.503 and 1.324, respectively. After the treatment of salanin, 17-Hydroxyazadiradione, Deacetyl gedunin, Gedunin and Dacetyl nimbin the LC_{50} and LC_{90} values were increased when compare to Azadirachtin. There was significant repellent activity after the treatment of neem products at three different concentrations (0.2, 0.4, and 0.6). Neem oil had higher repellent activity (<300 minutes at 0.6 concentration), followed by Gedunin which showed less activity (<200 minutes at 0.6 concentration). However, the ethanol applied arm served as control which provided maximum 7.0 minutes repellency. The leaves and pods were also tested smoke repellency bioassay. Smoke

*Corresponding author e-mail: kmvvkg@gmail.com, Mobile: +91 9894832849

emerged from the leaves and pods greater knock down effect were evident and percentage of repellency on leaf 59%, pod 53% and with commercial coil as positive control which showed 65% repellency. Moreover, smoke exposed larvae laid minimum number of eggs and hatchability also affected. From this study it has been concluded that based on the larvicidal, smoke repellent properties neem products showed mosquitocidal properties. Hence, it can be concluded that neem as effective biopesticides for the Integrated Vector Control Program. Moreover, neem is indigenous, medicinal importance and much of social relevance to people and traditional knowledge of our country.

Keywords: Neem products, larvicide, repellent, smoke toxicity, malarial vector.

INTRODUCTION

Mosquitoes are the prime vectors responsible for transmission of diseases to more than 70 billion people annually worldwide. As per the reports of World Health Organization, malaria alone kills 30 million people annually. Mosquitoes transmit the arbo-viruses which cause yellow fever, dengue hemorrhagic fever, epidemic polyarthritis, and several forms of encephalitis eg. Bancroftian filariasis which is caused by a nematode transmitted by mosquito bite (Mark and Fradin, 1998). *Anopheles stephensi* Liston (Diptera: Culicidae) is a major vector in India as well as in some of the West Asian countries and has been shown to be directly responsible for about 40-50% of the annual malarial incidence (Curtis, 1994; Collins and Paskewitz, 1995). Globally, malaria kills 3 million people each year, including 1 child every 30 seconds (Shell, 1997). Plant product has been used by traditionally human communities many part of the world against the vectors and species of insects. The phyto-chemicals derived from plant sources can act as larvicides, insect growth regulators, repellent and ovipositional attractants and have deterrent activities observed by many researchers (Babu and Murugan, 1998).

In India, bioactivity of 344 botanical agents (Sukumar *et al*., 1991) and active chemicals from neem were tested against mosquitoes (Schmutterer 1988, 1990). *Melia azedarach* L. and *Azadirachta indica* (Sapindales: Meliaceae), commonly known as Chinaberry or Persian lilac tree, are deciduous trees that are native to northwestern India; and have long been recognized for their insecticidal properties. These trees typically grow in the tropical and subtropical parts of Asia, but now-a-days they are also cultivated in other warm regions of the world because of their considerable climatic tolerance. Fruit extracts of *Melia azedarach* and *Azadirachta indica* elicit a variety of effects in insects such as antifeedant, growth retardation, reduced fecundity, moulting disorders, morphogenetic defects, and changes of behavior (Schmidt *et al.*, 1998; Abou Fakhr Hammad *et al.*, 2001; Gajmer *et al.*, 2002; Banchio *et al.*, 2003; Wandscheer *et al.*, 2004). Protection from arthropod bites is best achieved by avoiding or destroying infested habitats, wearing protective clothing, and using insect repellents. Repellents that are currently available in the market are either synthetic chemicals, such as N, N-diethyl-meta-toluamide (DEET), picaridin, ethyl butyl acetyl aminoproprionate or plant derived chemicals such as Citronella oil, oil of lemon eucalyptus. These insect repellent products are available in various types of formulation as sprays, sticks, foams, and lotions (Fradin and Day 2002). Mosquito control and personal protection from mosquito bites are currently the most important measures to control these diseases. The use of repellents is an obvious practical and economical means of preventing the transmission of these diseases to humans. The most common mosquito repellent formulations available on the market contain deet (*N, N*-diethyl-3-methylbenzamide), which has shown excellent repellency against mosquitoes and other biting insects (Yap 1986, Coleman *et al.*, 1993).

Recently, extracts of several plants including neem (*Azadirachta indica* A. Juss), Citronella grass (*Cymbopogan nardus* Rendle), basil (*Ocimum basilicum* L., *Ocimum gratissimum* L., *Ocimum americanum* L.), clove (*Syzygium aromaticum* L.), prickly straggler (*Solanum trilobatum* L.), musk basil (*Moschosma polystachyum* L.) and thyme (*Thymus vulgaris* L.) have been studied as possible mosquito repellents (Chokechaijaroenporn *et al.*, 1994; Suwonkerd & Tantrarongraj, 1994; Rajkumar & Jebanesan, 2004, 2005). Neem trees, (*Azadirachta indica*) native of India, belonging to family Meliaceae are fast growing evergreen trees 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 (National Research Council, 1992). Neem seeds contain approximately 99 biologically active compounds of which azadirachtin, nimbin, nimbidin and nimbolides are major molecules. Many of these derived products have antifeedancy, ovicidal activity, fecundity suppression besides insect growth regulation and repellency against insects (Isman, 2006, Schmutterer, 2002).

Neem products have low toxicity to birds, fish and mammals and are less likely to induce resistance due to their multiple mode of action on insects. In addition to this, insect growth regulatory activity of neem weakens the cuticle defence system of the larvae causing easy penetration of pathogenic organisms into insect system. 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 (Su and Mulla, 1998, Su and Mulla, 1998). Chemical investigation on the products of the neem tree was extensively undertaken in the middle of the twentieth century. Since the early report by (Siddiqui, 1942) in 1942 on the isolation of nimbin, the first bitter compound isolated from neem oil, more than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural *diversity* of these compounds (Koul *et al.*, 1990, Chatterjee and Pakrashi, 1994, Mitra, 1963, Warthen, 1979, Taylor, 1984, Champagne, *et al.*, 1992, Kraus, 1995, Devakumar and Sukh Dev, 1996, Govindachari, 1992). The compounds have been divided into two major classes: isoprenoids and others (Kraus, 1995). The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and Csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. The details of the chemistry of various compounds falling under these groups have already been reviewed (Kraus, 1995, Devakumar and SukhDev, 1996).

In this group of neem products are capable of inflicting multiple effects on insects such as antifeedant, growth regulation, fecundity, suppression and sterilization, oviposition, repellency or attractancy and changes in biological fitness. Neem (*Azadirachtica indica* A.Juss) is parahaps the most useful traditional pesticidal plant in India. Each part of the neem tree has insecticidal properties (Murugan *et al.*, 1995; Murugan and Jeyabalan, 1995; Murugan *et al.*,1999).Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of *A. indica* demonstrated several biological activities. From this crude principle some tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated (Siddiqui, 1942, Mitra *et al*., 1971).The spermicidal activity of nimbidin and nimbin (1) was reported in rats and human as early as 1959 (Sharma and Saksena, 1959). Nimbidin also demonstrated antifungal activity by inhibiting the growth of *Tinea rubrum* (MurthySirsi, 1958). . Nimbolide (2) has been shown to exert antimalarial activity by inhibiting the growth of *Plasmodium falciparum* (Rochanakij, *et al*., 1985, Khalid, *et al*., 1989). Nimbolide also shows antibacterial activityagainst *S. aureus* and *S. coagulase* (Rojanapo, *et al*., 1985). Gedunin (3), isolated from neem seed oil has beenreported to possess both antifungal (Rao, *et al.*, 1986) and antimalarial(Khalid, *et al*., 1989) activities. Azadirachtin (4), highly oxygenated C-secomeliacins isolated from neem seed and having strong antifeedant activity (Kraus, 1995, Govindachari, 1992, Kirtikar and Basu, 1975).

Repellent action of neem oil in the form of mats (Sharma *et al*., 1993) and neem cream (Dua *et al*., 1995) have been evaluated against mosquitoes. Benzene and methanol extracts of *Artemisia vulgaris* have been reported to have repellent activity against *Ae. aegypti* (Yit *et al*., 1985). Two types of botanical insecticides can be obtained from seeds of the Indian neem tree, *Azadirachta indica* (Meliaceae) (Schmutterer, 2002). Neem oil, obtained by cold-pressing seeds, can be effective against soft-bodied insects and mites but is also useful in the management of

phytopathogens. Apart from the physical effects of neem oil on pests and fungi, disulfides in the oil likely contribute to the bioactivity of this material. More highly valued than neem oil are medium-polarity extracts of the seed residues after removal of the oil, as these extracts contain the complex triterpene azadirachtin. Neem seeds actually contain more than dozen azadirachtin analogs, but the major form is azadirachtin and the remaining minor analogs likely contribute little to overall efficacy of the extract. Seed extracts include considerable quantities of other triterpenoids, notably salannin, nimbin, and derivatives thereof. The role of these other natural substances has been controversial, but most evidence points to azadirachtin as the most important activeprinciple (Isman *et al*., 1996). (Neem oil Limonoids) is a trade marked natural extract from Sabinsa Corporation, obtained from cold pressed neem seed oil and standardized to contain not less than 50% Total Limonoids and 1000 ppm Azadirachtin. Potential cosmeceutical applications include antibacterial, antifungal, antiparasitic, insect repellant, anti-pediculosis formulations for topical use in skin and hair care. (Neem Oil Limonoids: Product Overview, 2007). The present paper is to investigate on the larvicidal, pupicidal, smoke repellency effect of neem products against malarial vector, *Anopheles stephensi.*

METHODS

1. Collection of eggs

The eggs of *Anopheles stephensi* were collected from local (in and around Coimbatore, India) different breeding habitats with the help of a 'O' type brush. The eggs were then brought to the laboratory and transferred to 18 x 13 x 4 cm size enamel trays containing 500 ml water and kept for larval hatching. They were hatched and reared have been still maintained from many generations in the laboratory. The eggs and larvae obtained from this stock were used for different experiments.

2. Maintenance of larvae

The larvae reared in plastic cups. They were daily provided with commercial fish food (Lyimo *et al*., 1992). Water was changed alternate days. The breeding medium was regularly checked and dead larvae were removed at sight. The normal cultures as well as breeding cups used for any experimental purpose during the present study were kept closed with muslin cloth for preventing contamination through foreign mosquitoes.

3. Maintenance of pupae and adult

The pupae were collected from culture trays and were transferred to glass beakers containing 500 ml of water with 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 sides (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 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 they were provided an animal for blood feeding.

4. 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 was suggested (Judson 1967 and 53Briegel 1990). Both females and males were provided with 10% glucose solution as described (Villani *et al*., 1983) on cotton wicks. The cotton was always kept moist with the solution and changed every day. An egg trap (cup) lined with filter paper containing pure water was always placed at a corner of the cage. This arranged made collection of eggs easier.

5. Colonization of mosquito species

Three species of mosquito as malarial vector, *Anopheles stephensi* were collected and maintained at the laboratory by standard procedures. The maintenance of the larvae, pupae and adult has done at the standard procedures at the laboratory. Collection and culture of brackish water mosquitoes were collected and maintained at the laboratory for further laboratory and field bioassay.

6. Neem Limonoids

The neem oil which was procured from the Local Neem Oil Mill, Kalverrampalayam, Coimbatore-641 046, India. Six limonoids (purity 99%), Six

neem limonoids (purity>99%), namely azadiractin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin were sent from Central Research Laboratories, Taiyo Kagaku Co Ltd., Japan. They were dissolved in isopropanol and different concentrations were prepared by dilution with isopropanol.

7. Larvicidal Activity

Laboratory colonies of mosquito larvae were used for the larvicidal activity. Twenty-five numbers of fourth instar larvae were introduced into the 500 ml glass beaker containing 249 ml of de-chlorinated water and 1 ml of desired concentrations of neem products was added separately. Larval food was given for the test larvae. At each tested concentration 3 trials were made and each trial consisted of five replicates. Mixing 1ml of acetone with 249 ml of de-chlorinated water set up the control. In the plant extracts the larvae exposed to de-chlorinated water without acetone served as control. The control mortalities were corrected by using Abbott's formula (Abbott's, 1925).

$$\text{Percentage mortality} = \frac{\text{Number of dead pupae}}{\text{Number of pupae introduced}} \times 100$$

LC_{50}, LC_{90}, regression equation and 95% confidence limit of lower confidence of limit (LCL) and Upper Confidence Limit (UCL) were calculated from toxicity data by using probit analysis (Finney, 1971).

8. Repellency tests

The neem products were evaluated for their repellent activities against *An. stephensi* using the human – bait technique (Fradin and Day, 2002). First, 1 ml neem oil was mixed with 5 ml coconut oil and diluted in ethanol and prepared 2, 4 and 6% concentrations. For the test, 20 disease free, laboratory-reared female mosquitoes were placed into separate laboratory cages (45 x 38 x 38 cm). Before the test, the volunteer's skin was washed with unscented soap and the tested essential oil was applied from the elbow to the finger tips. In each cage one arm was inserted for one test concentration and the other arm applied with ethanol served as control. The treated and control arms were interchanged regularly to eliminate bias. Each test concentration was repeated five times and in each replicate subject different volunteers to nullify any effect of colour of the skin on repellent. Volunteers were asked to follow the testing protocol.

Volunteers conducted their test of each concentration by inserting the treated and control arms alternatively into a same cage for one full minute for every five minutes. If they were not bitten within 20 minutes, then the arms were reinserted for 1 full minute for every 15 minutes, until the first bite occurred.

9. Smoke Toxicity Test

Cassia occidentalis parts were used for smoke toxicity assay. The mosquito coil was prepared by following method of Saini *et al*. (1986) with minor modifications by using 4 grams from powdered plant sample considered as active ingredient and 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 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 was Mortein coil which was 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 *Anopheles stephensi* was calculated in terms of percentage of unfed mosquitoes due to treatment.

$$= \frac{\text{Number of unfed mosquitoes in treatment - Number of unfed mosquito in control1}}{\text{Number of mosquitoes treated}} \times 100$$

The live blood fed mosquitoes were reared in a 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 raisins and a 5% sucrose solution soaked in cotton balls were provided as a food source. Water containing powdered yeast and dog biscuits were also kept inside the cage in a glass bowl for oviposition. The eggs from the cage were collected daily till all the mosquitoes died. A total 50- 100 eggs were allowed to hatch in plastic trays measuring 30 x 25 x 6 cm, containing about 2.5 liters of unchlorinated tap water. Hatched larvae's 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. Survival and dead instars were counted and reduction in the population from the smoke treated mosquitoes was calculated using the formula.

$$Population\ reduction(\%) = \frac{\text{Number of larvae hatched in control - Number of larvae hatched in treated}}{\text{Number of larvae hatched in control}} \times 100$$

10. Statistical Analysis

The data gets from the bioassay subject to statistical analysis. The SPSS software package was computing all the data including probit analysis, correlation co-efficient and mean of the sample.

RESULTS

The neem products also had significant larvicidal activity. The larval mortality was dose dependant. The LC_{50} and LC_{90} values of Azadirachtin treatment against *Anopheles stephensi* at 0.50, 1.0 and 1.5 ppm concentration was 0.299% and 1.061%, respectively. After treatment of neem oil at 0.50, 1.0 and 1.5 ppm concentration was 0.503 and 1.324, respectively. Similarly, Salannin was 0.438% and 1.420%, 17-Hydroxyazadiradione was 0.387% and 1.553%, Gedunin was 0.558% and 1.568%, Deacetylgedunin was 0.461% and 1.384, Deacetylnimbin was 0.594% and 1.542%, respectively.After the treatment of salanin, 17-Hydroxyazadiradione, Deacetyl gedunin, Gedunin and Dacetyl nimbin the LC_{50} and LC_{90} values were increased when compare to Azadiractin. The regression equation of neem oil for 4th instar were X= 1.560, Y= -0.784, azadirachtin were X= 1.682, Y= -0.502, 17-Hydroxyazadiradione were X= 1.100, Y= - 0.426, Salannin were X= 1.305, Y= - 0.571, Deacetyl gedunin were X= 1.387, Y= - 0.639, Dacetyl nimbin were X= 1.353, Y= - 0.804 and Gedunin were X= 1.269, Y= - 0.639, respectively.

The repellent activity of neem products were tested at three different concentrations (0.2, 0.4, and 0.6) of these the neem product of neem oil, Azadirachtin, Salannin, 17-Hydroxy-azadiradione, Deacetyl gedunin, Dacetyl nimbin and Gedunin exhibited relatively. The neem oil complete protection time at 0.2% concentration, the production time was 165 mts, at 0.4% the protection time was 248 mts, at 0.6% the production time was 356 mts. Similarly, Azadirachtin at 0.2% was 134 mts, at 0.4% was 237 mts, at 0.6% was 348 mts, Salannin at 0.2% was 123mts, at 0.4% was 220mts, at 0.6% was 313mts, 17- Deacetyl gedunin at 0.2% was 119mts, at 0.4 %was 210 mts, at 0.6% was 304mts, Dacetyl nimbin at 0.2% was 105 mts, at 0.4% was 198 mts, at 0.6% was 289 mts, Gedunin at 0.2% was 95 mts, at 0.4% was 152 mts, at 0.6% was 206 mts, respectively. The neem oil which showed high repellent effect (<300 minutes at 0.6 concentration), followed by Gedunin which showed less (<200 minutes at 0.6 concentration). However, the ethanol applied arm served as control provided maximum 7.0 minutes repellency in this study. The leaves and pods were also tested smoke repellency bioassay. Smoke emerged from the leaves and pods of had potential knock down effect and percentage of repellency on leaf 59%, pod 53% and with commercial coil as positive control which showed 65% repellency, respectively. Table 4 shows the result of smoke toxicity effect of different parts of (leaves and pods) Neem ensured population of *A. stephensi*. The numbers of eggs laid by the alive, fed females were shown. Number of eggs laid and the hatchability were greatly reduced or affected by the exposure of smoke from Neem. The percentage reduction of hatchability by the smoke from leaves showed 57.4 % and from the pods showed 59.2%. The leaves showed a significant effect on the fecundity and hatchability.

Table 1. Larvicidal activity of neem products against 4th instar larva of malarial vector, *Anopheles. stephensi.*

Neem products	Mortality (%) (Mean ± SE)	Regression equation	Chi square (χ^2)	LC_{50} (Fiducial limits) ($\mu g/cm^2$)	LC_{90} (Fiducial limits) ($\mu g/cm^2$)
Azadirachtin	62.6 ± 0.5 89.82 ± 0.3 97.4 ± 0.5	X= 1.682 Y= -0.502	0.632	0.299 (0.036) (0.452)	1.061 (0.940) (1.245)
17-Hydroxyazadiradione	56.2 ± 0.7 72.6 ± 0.9 90.0 ±0.7	X= 1.100 Y= - 0.426	0.479	0.387 (0.034) (0.576)	1.553 (1.339) (1.967)
Salannin	53.6 ± 1.1 76.2 ± 0.9 92.6 ± 0.5	X= 1.305 Y= - 0.571	0.039	0.438 (0.177) (0.594)	1.420 (1.249) (1.714)
Deacetyl gedunin	52.0 ± 0.7 77.6 ± 0.9 92.4 ± 0.9	X= 1.387 Y= - 0.639	0.100	0.461 (0.228) (0.606)	1.384 (1.226) (1.649)
Neem oil	51.4 ± 1.0 75.2 ± 0.8 95.2 ± 0.8	X= 1.560 Y= -0.784	0.843	0.503 (0.314) (0.628)	1.324 (1.183) (2.548)
Gedunin	48.0 ± 0.7 69.4 ± 0.6 89.2 ± 0.8	X= 1.269 Y= - 0.639	0.265	0.558 (0.339) (0.699)	1.568 (1.377) (1.903)
Dacetyl nimbin	46.4 ± 1.2 68.0 ±1.2 90.2 ± 0.5	X= 1.353 Y= - 0.804	0.632	0.594 (0.403) (0.723)	1.542 (1.364) (1.844)

Table 2. Repellent activity of neem products against malarial vector, *An. stephensi.*

Neem products	Concentration (%)	Complete – protection time (min)	
		Treated	Control
Neem oil	0.2	165.0 ±1.0[a]	6.0 ± 1.0
	0.4	248.0 ± 1.1[a]	4.0 ± 1.0
	0.6	356.0 ± 1.4[a]	5.0 ± 1.0
Azadirachtin	0.2	134.0 ± 1.3[b]	5.0 ±1.0
	0.4	237.0 ± 1.2[b]	7.0 ±1.0
	0.6	348.0 ± 0.7[a]	4.0 ±1.0
Salannin	0.2	123.0 ± 1.0[c]	5.0 ±1.0
	0.4	220.0 ± 0.7[b]	6.0 ±1.0
	0.6	313.0 ± 0.4[a]	5.0 ±1.0
Deacetylgedunin	0.2	119 ± 0.7[a]	5.0 ±1.0
	0.4	210 ± 0.7[a]	6.0 ±1.0
	0.6	304 ± 1.0[b]	5.0 ±1.0
17-Hydroxyazadiradione	0.2	109 ± 0.4[a]	6.0 ±1.0
	0.4	201 ± 0.3[a]	4.0 ±1.0
	0.6	307 ± 0.4[a]	5.0 ±1.0
Deacetylnimbin	0.2	105 ± 0.7[a]	5.0 ±1.0
	0.4	198 ± 1.3[b]	5.0 ±1.0
	0.6	289 ± 0.7[a]	6.0 ±1.0
Gedunin	0.2	95.0 ± 1.1[b]	4.0 ± 1.0
	0.4	152.0 ± 1.0[b]	4.0 ± 1.0
	0.6	206.0 ± 0.7[a]	6.0 ± 1.0

Each value (mean ± SE) represents mean of five values. Within a column means followed by the same letter (s) are not significantly different at 5% level by DMRT.

Control I *: ethanol.

*Corresponding author e-mail: kmvvkg@gmail.com, Mobile: +91 9894832849

Table 3. Smoke toxicity effect of leaves of neem against biting activity of *Anopheles stephensi.*

Neem parts used in grams	No. of mosquito tested	Fed mosquito	Unfed mosquito		Total	% unfed over control I
			Alive	Dead		
Leaf 2 G	100	16b	36	48	84	59
Pods 2 G	100	22c	42	36	78	53
Control I *	100	75	25	0	25	0
Control II *	100	10a	47	43	90	65

Within a column means followed by the same letter (s) are not significantly different at 5% level by DMRT.
Control I *: Negative control – blank without plant material.
Control II *: Positive control – mortein coil.

Table 4. Smoke toxicity effect of leaves of *Azadirachta indica* on reproduction and survival of *Anopheles stephensi.*

Azadirachta indica parts used	No. of mosquito tested	Total No. of eggs	Total No. of larva hatched from the eggs	% of reduction in population over control I
Leaf	25	1045	534	57.4
Pod	25	950	512	59.2
Control I *	25	1450	1256	0
Control II *	25	820	386	69.2

Control I * : Negative control – blank without plant material.
Control II *: Positive control – mortein coil.

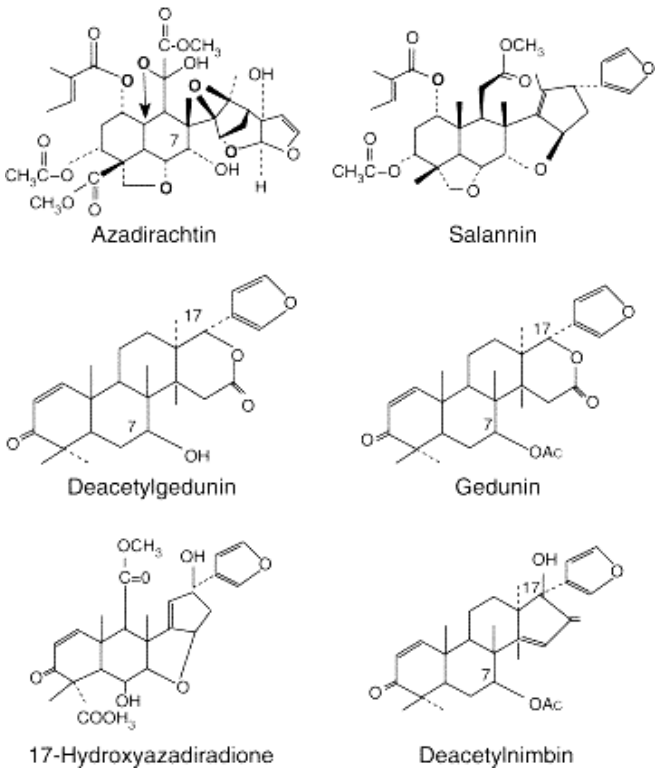

Figure 1. Structure of neem limonoid.

DISCUSSION

Malaria is the largest single component of disease burden; epidemic malaria, in particular, remains a major public health concern in tropical countries. In many developing countries, and especially in Africa, malaria exacts an enormous toll in lives, in medical costs, and in days of labor lost (Lambert, 2005). Since from the ancient years the plants were being used for the control of mosquitoes in many ways like burning the plant parts, keeping the plants near window etc., In the present study also we have shown that neem product has larvicidal as well as repellent activity against various species of mosquitoes. Recently, strong repellent actions of *Azadirachata indica, Cymbopogan martinii var. sofia, C.citratus, C.naridus, Ocimum spp* etc. have been reported against *An. culicifacies* and other species of mosquitoes (Ansari and Razdan, 1994, 1995; Sharma et al., 1993; Sharma and Ansari, 1994). These findings have re-emphasized the need to explore the possibility of using herbal-based repellents (Osmani *et al.*, 1972) as supplementary and complimentary measures for malaria control. This will reduce the chemical burden on the environment. The neem oil more potential effect against mosquito larvae. Murugan *et al.* (1999) (established the neem seed kernel extract possess anti-pupational property for mosquito species. Babu and Murugan (1996) investigated that the larvicidal effect of resinous exudate from the tender leaves of *Azadirachta indica.* Vahitha *et al*. (2002) studied the larvicidal efficacy of *Pavonia zeylamica* L. and *Acacia ferruginea* D.C. against *Culex quinquefasciatus* Say. The present study showed that, after the treatment of salanin, 17-Hydroxyazadiradione, Deacetyl gedunin, Gedunin and Dacetyl nimbin, the LC_{50} and LC_{90} values were increased when compare to Azadirachtin. The most active constituent, azadirachtin, a triterpenoid, has show to have properties including feeding and ovipositional deterrence, repellency, growth disrurption, reduced fitness and sterility in a number of species of hemimetabolous and holometabolous insects (Ascher and Meisner, 1989; Schmutterer, 1990). From the light of literature we came to know about many larvicidal studies conducted using plants and results clearly suggested that the *C. inerme* interfered with developmental processes of the fourth instar larvae and pupae of *A. aegypti*. In this context, the observations that exposure of fourth instar mosquito *Culex quinquefasciatus* to ether extract of *C. inerme* leaves resulted in death at larval–pupal molt and pupal–adult eclosion and suggesting inhibition of the moulting process (Pereira and Gurudutt, 1990) lend further support to our observations.

Lavie *et al.* (1967) reported that isolated gedunin and 7-deacetyl gedunin from neem seed oil and bark and 7-benzoyl derivative from dried seeds. Gedunin was shown to posses both antifungal and antimalarial properties. The antifungal and antimalarial properties may be effected to the malarial vector, *Anopheles stephensi*. Dhar *et al.* (1996) demonstrated that the inhibitory effect of neem oil volatiles on gonotropic cycle in *An. stephensi* and *An. culicifacies*. A neem oil formulation containing 32% neem seed oil (an equivalent of 0.03% azadirachtin), an emulsifier (5%) and 63% iso propanol (solvent) was investigated for its larvicidal activities against *An. gambiae* (Okumu, *et al.,* 2007). It was toxic to mosquito larvae with LC_{50} value of 11 ppm and also reported to possess insect growth regulators. Gianotti and co workers (Gianotti, *et al.,* 2008) used powdered seeds of neem trees and applied twice a week to known breeding sites for *An. gambiae* at the rate of 10 gm/m^2 of pool surface area for effective larval control. Azadirachtin acts as anti-ecdysteroid and kills larvae by growth inhibition effect (Zebit, 1984). In the present investigation, neem products was found effective to control mosquito larvae in laboratory conditions and more than 90% reduction of *Anopheles stephensi* larvae were observed up to three weeks of post application. Limonoid compounds are found in almost all parts of the neem plant. The active ingredients in Neem extracts are very low in toxicity and thus are not toxic to mammals and also quickly biodegrade from the sun's light. However, the use of Azadirachtin based neem pesticides may be limited by the acid and base sensitivity of the compound and its susceptibility to photo degradation due to presence of light-absorbing moieties (Stokes and Redfern, 1982; Barnby et al., 1989). Hence, the neem oil not effected to human skin and not makes allergic reaction to human. The neem oil affected to target organism malarial vector, *Anopheles stephensi* only. Subramonia Thangam and Karthiresan (1992) stated that smoke from burning various dry materials has been used since early times to deter insects, especially mosquitoes and studied smoke repellency effect of marine plants against *Culex quinquefasciatus.*

*Corresponding author e-mail: kmvvkg@gmail.com, Mobile: +91 9894832849

(Schmutterer (1990) stated that when an insect eats azadirachtin, it actively attacks the insect's reproductive cycle, its feeding pattern, its bodily development, as well as acting direct toxin. Thus, when azadirachtin is sprayed on the plant and the insect take a bite – if it can stand the bitter taste – the insect will no longer be able to reproduce, eat and grow. Similarly, The adverse effect of smoke exposure on adult mosquito which prevents egg laying and hatching may be due to interference of endocrine events causing reproductive malfunction.

CONCLUSION

From this study it has been concluded that based on the larvicidal, smoke repellent properties neem products showed mosquitocidal properties. Hence, it can be concluded that neem as effective biopesticides for the Integrated Vector Control Program. Moreover, neem is indigenous, medicinal importance and much of social relevance to people and traditional knowledge of our country.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this article.

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