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A comparative study of toxicity evaluation and effects of *Moringa oleifera* Lam. flowers extract on polytene chromosome of vector (*Anopheles stephensi* L.) and non vector (*Anopheles annularis* W.) anopheline mosquito fauna

[1] Azra Parveen, [2] Archana Gaur, [3] Arti Prasad

[1, 2] Insect Microbial and Herbal Control Laboratory, Department of Zoology, M.L.S.U, Udaipur, Rajasthan, India

[3] Professor and Head, Department of Zoology, Insect Microbial and Herbal Control Laboratory, Department of Zoology, M.L.S.U, Udaipur, Rajasthan, India

Abstract
The natural plants products provide number of associated benefits that bound pesticides environmental impact due to eco friendly, cost-effective and target specific nature and may be useful in preventing resistance developed in mosquito population. Keeping this fact in mind effects of methanolic extracts of *Moringa oleifera* Lam. flowers on polytene chromosomes of vector (*Anopheles stephensi* L.) and non vector (*Anopheles annularis* W.) anopheline mosquitoes were studied and compared both for dose mortality assessment and visible changes. The larval mortality was observed at 24hrs and 48 hrs of time exposure and LC_{50}, LC_{90} and probit equation were calculated. The LC50 and LC90 values obtained for *Moringa oleifera* flowers extract against *Anopheles annularis* were 47.05mg/l, and 95.61.50mg/l respectively at 48 h of exposure time. And Same for *Anopheles stephensi* were 80.46 mg/l and 130.14 mg/l. Dose mortality test observation showed that *Anopheles stephensi* mosquitoes are more resistant as compared to *Anopheles annularis.* It was also reported that due to the effects of flowers extract, chromosome become diffused, super contracted or clumped in greater intensity in *Anopheles annularis* as compared to *Anopheles stephensi.*

Keywords: *anopheles stephensi*, plant extract, polytene chromosomes, *moringa oleifera*, stickiness etc.

1. Introduction
Malaria remains a critical problem in global public health. It is estimated that malaria is responsible for nearly 110 million clinical cases and an estimated 300,000 deaths per year; the disease is responsible for 25% infant mortality, 30% childhood mortality and is associated with 11% maternal deaths. WHO [1] gave its priority to control vector rather than parasites as it can only give a sustainable solution for vector borne diseases in countries like India & Africa. Anopheles are responsible for transmitting malaria in humans of which some species are carriers known as vector but some does not transfer the malaria parasite, these are called non vector. Malaria vector and non-vectors may periodically extend beyond their normal area of distribution when temporary suitable conditions occur in neighbouring areas [1].

.*Anopheles stephensi* Liston (Diptera: Culicidae) is an important malaria vector in the Persian Gulf and South Iran [2], in urban areas of the Indian subcontinent [3], as well as in rural areas of North Pakistan and East Afghanistan [4]. This species is also an outstanding laboratory model system for malaria parasite transmission studies [5]. The tax on *An. Annularis* is reported to be a species complex comprising two sibling species viz. A and B in India. From past literature it was found that in Southern Rajasthan the species B is reported as non-vector.

In the last 50 years, vectors have mainly been controlled with synthetic Insecticides, but has met with tremendous setbacks in the light of the development of vector resistance and some attendant environmental hazards. Thus the major drawback with the use of these synthetic insecticides is that these are non-selective, harmful and adversely affect the other organisms in the environment [6]. The above constraints have resulted in an urge to look for environmental friendly, cost effective, biodegradable and target specific insecticides. Plant products provide one of the best platforms where all such qualities of a vector control agents can be fulfilled.

In our study we have selected *Moringa oleifera* (flowers) as a biopesticide. Plant products have been used traditionally in many parts of the world against the vectors borne diseases**.** Biopesticides explored from plant sources can act as larvicides and can be responsible for the interruption of the transmission of mosquito borne diseases at the individual as well as at the community level [7].

Cytogenetics has proven immensely useful for differentiating among sympatric taxa and chromosomal forms. In fact cytogenetics remains as the only tool to reliably differentiate between all mosquitoes chromosomal forms. Previous studies with various mosquito species have demonstrated that mosquito host feeding behaviours and vector competence to malaria parasites are under genetic control [8, 9]. Thus the study of mosquito cytogenetic is important in medical field considering the variety of disease they transmit. The uses of cytogenetic provide the necessary background knowledge about the behaviour of these vectors, so it can help in controlling them.

Anopheline mosquitoes, like Drosophila, are renowned for the

presence of polytene chromosomes [10]. These giant chromosomes were first described in larval chironomids by Balbiani [11] but only accorded proper significance 50 years later by Heitz & Bauer [12].

The polytene chromosomes of Dipteran salivary glands offer a natural system in which differential gene activity can be analysed unswervingly at the level of the genes themselves. The study on morphology and development of salivary gland and their chromosome was done by Rishikesh [13] and Moreira [14]. A literature survey showed several reports on the study of the polytene chromosomes in *Culex quinquefasciatus* [15-19].

These chromosomes are characterized by nuclei with giant chromosomes which are distinct and can be easily observed under light microscope. As a result, the effects of plant extract were clearly identified. Biopesticides are an interesting alternative for insect pest control due to the properties of cost effectiveness, ecofriendly nature and easily degradable on non-target population. Thus the comparative susceptibility of karyotype profile of vector (*Anopheles stephensi*) and non-vector (*Anopheles annularis*) anopheline mosquito against herbal biopesticide is the main objective of this study.

2. Material and methods

2.1 Collection of plant

Mature fresh flowers of *Moringa oleifera* plant were collected from nearby areas like Mohanlal Sukhadia University campus and other nearby regions of Udaipur (Raj.), India. After washing the flowers they were shade-dried, and finely powdered in a mixer-grinder.

2.2 Extraction

The flowers powder (10g/ solvent) was loaded in Soxhlet apparatus[20]. The liquid were removed from the extract using a rotary vacuum evaporator to collect the crude extract.

2.3 Test organisms

Anopheles stephensi and *Anopheles annularis* mosquitoes were reared in Insect Microbial and herbal control laboratory, Department of Zoology, MLSU, Udaipur (Raj) India. Field collected larvae were reared to adult and identified for species and then pure culture was maintained in the laboratory as per WHO protocol.

2.4 Test for mortality and bioassay of larvae

The larvicidal activity of the methanolic plant extracts (*Moringa oliefera* flowers) against the larvae of *Anopheles stephensi* and *Anopheles annularis* mosquito was determined by the method recommended by WHO [21]. 1% stoke solution of methanolic extract of flowers was prepared. From this stock solution different doses in ppm concentration were prepared like20ppm, 40ppm, 60ppm, 80ppm and 100ppm. These concentrations were selected after the pre-test conducted. Batches of 30 forth-instars larvae of both *Anopheles stephensi* and *Anopheles annularis* were selected and transferred in a small plastic containers containing solution which we had prepared at different ppm concentration. Simultaneously control groups were also set in three replicas each with 30 larvae of same age. The whole experiment was set at the standard temperature and humidity maintained in the culture room of Insect microbial and herbal control laboratory, UCOS, MLSU, Udaipur (Raj).

The mortality observed (ppm) was corrected using Abbott's formula [22] during the observation of the larvicidal potentiality of the flowers extract. Statistical analysis of the experimental data was performed with MS EXCEL 2003 to find the LC50, LC90 and Probit equations.

$$Pr = \frac{Po - Pc}{100 - Pc} \times 100$$

Where,
Pr = Corrected mortality (%)
Po = Observed mortality (%)
Pc = Control mortality (%)

2.5 Dissection of salivary gland and preparation of slide

Regarding dissection and microscopic slide preparation we have followed the method explained by Anthony Cornel in the second edition [23] of "Methods in Anopheles research" which have explained the best methods for these techniques. Preparation of slides was done after the dissection of salivary gland from 4th instar larvae which remain alive after treatment and control. The salivary glands of larvae were dissected in 5% propionic acid under dissecting microscope. The dissected glands were fixed in carnoys fixative for 1 min followed by 50% propionic acid for three minutes until they became cleared and swollen to about twice their original size. Staining was done by 2% lacto aceto orcein for 4 minutes. The excess stain was removed with a piece of tightly rolled up absorbent paper. Mounting was done in 50% propionic acid and proper squash technique was applied. The sides of cover slip were painted with nail paint so that air may not pass in. The microscopic photography were done next day for good result and studied for visible changes. Jensen [24], Russel *et al.* [25] and Trembley [26] have also cited good literature about it.

3. Results

The extract of *Moringa oleifera* Lam. has been studied for use as ecofriendly insecticide instead of eco enemy synthetic insecticide. In the present investigation the *Moringa oleifera* flowers was selected and biological activity of these plants were examined to find out its potentiality and effectiveness against vector and non-vector anopheline mosquito larvae together with their polytene chromosome.

3.1 Dose-mortality assessment

When different doses of flowers extract of *Moringa oleifera* Lam. were applied on mosquito larvae, significant increase in mean mortality with increase in dose as well as time was observed. Mean mortality was directly proportionate to time as well as doses. At lower concentration of 20 ppm, the mean mortality came out to be 0.3 $\pm$0.58 and 0.7 $\pm$0.58 at 24 hours and 48 hours of exposure respectively. As concentrations were increased mean mortality also increased i.e. 1.3 $\pm$0.58, 3.7 $\pm$0.58, 9.0 $\pm$1.00 and 15.0 $\pm$1.00 at 40, 60, 80 and 100 ppm at 24 hours and 3.0 $\pm$1.00, 8.3 $\pm$0.58, 13.3 $\pm$1.53 and 19.7 $\pm$2.52 at 40, 60, 80, 100 ppm at 48 hours against 0.00±0.00 and 0.3 $\pm$0.58 in control respectively (Table 1).

And for *Anopheles annularis* at lower dose of 20ppm, mortality was 4.7 $\pm$1.15 whereas at highest concentration of

100ppm, mortality reached to 17.3 ±3.51 at 24 hours of exposure. Further at 48 hours of exposure time the mortality increased from 11.0 ±1.00 to 28.0 ±2.00 from 20ppm to 100ppm respectively against control mean mortality of 0.00±0.00 at 24 hours and 0.3 ±0.58 at 48 hours of exposure respectively (Table 2). Graphical representation of the dose and mortality relationship also justified the above observations showing gradual increase in mortality with increase of concentrations as well as time (Fig 1 & 2).

The result of Probit equation against *Anopheles stephensi* was -1.960 + 0.024 and for *Anopheles annularis* it was -1.242 + 0.026 (Table 3).

Table 1: Efficacy of Flowers extract of *Moringa oleifera* Lam. against *Anopheles stephensi* (L.) at 24 and 48 hrs

Dose (ppm)	Time	
	24hr	48hr
20	0.3 ±0.58	0.7 ±0.58
40	1.3 ±0.58	3.0 ±1.00
60	3.7 ±0.58	8.3 ±0.58
80	9.0 ±1.00	13.3 ±1.53
100	15.0 ±1.00	19.7 ±2.52
Control	0.0	0.3 ±0.58

Table 2: Efficacy of flower extract of *Moringa oleifera* Lam. against *Anopheles annularis* (W.) at 24 and 48 hrs

Dose (ppm)	Time	
	24hr	48hr
20	4.7 ±1.15	11.0 ±1.00
40	8.7 ±1.15	14.3 ±1.53
60	11.7 ±1.53	18.0 ±2.65
80	14.3 ±3.06	22.7 ±2.52
100	17.3 ±3.51	28.0 ±2.00
Control	0.0	0.3 ±0.58

Table 3: Probit Equation

Moringa oleifera Lam. flowers against *Anopheles stephensi* (L.)	Probit = -1.960 + 0.024 (Dose)
Moringa oleifera Lam.flowers against *Anopheles annularis* (W.)	Probit = -1.242 + 0.026 (Dose)

Table 4: Toxicity of flower extract (LC_{50} and LC_{90}) of *Moringa oleifera* Lam. against *Anopheles stephensi* (L.) and *Anopheles annularis* (W.) mosquitoes after 48 hr exposure time

1. ***Anopheles stephensi* (L.)**	**LC_{50} value in 48 hr**	**LC_{90} value in 48 hr**
Moringa oleifera Lam. flowers extract	80.46	130.14
2. *Anopheles annularis* (W.)		
Moringa oleifera Lam. flowers extract	47.05	95.61

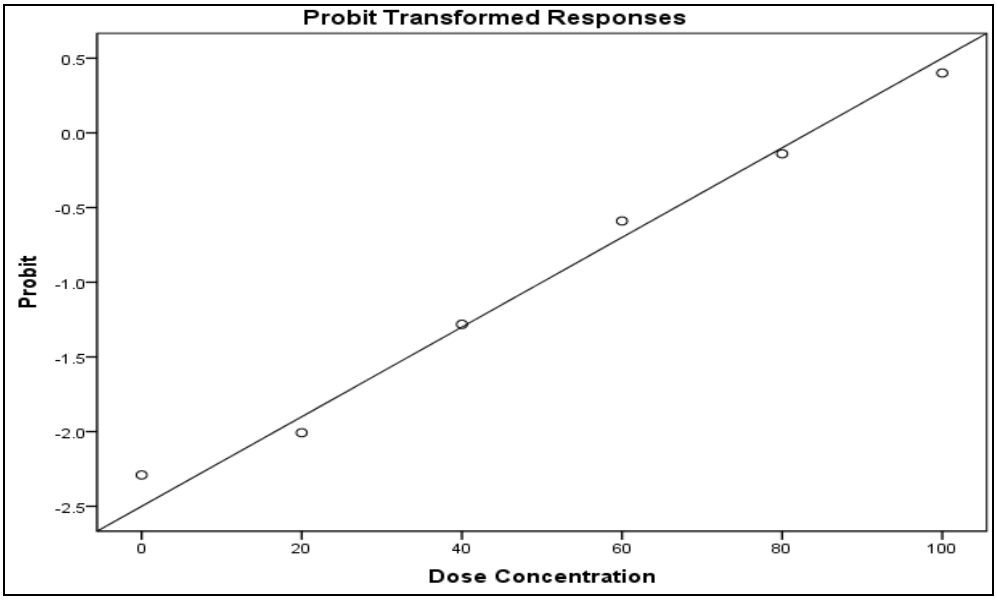

Fig 1: Probit transformed responses for concentrations v/s mortality against fourth instar larvae of *Anopheles stephensi* (L.) treated with *Moringa oleifera* Lam. flowers extract after 48h

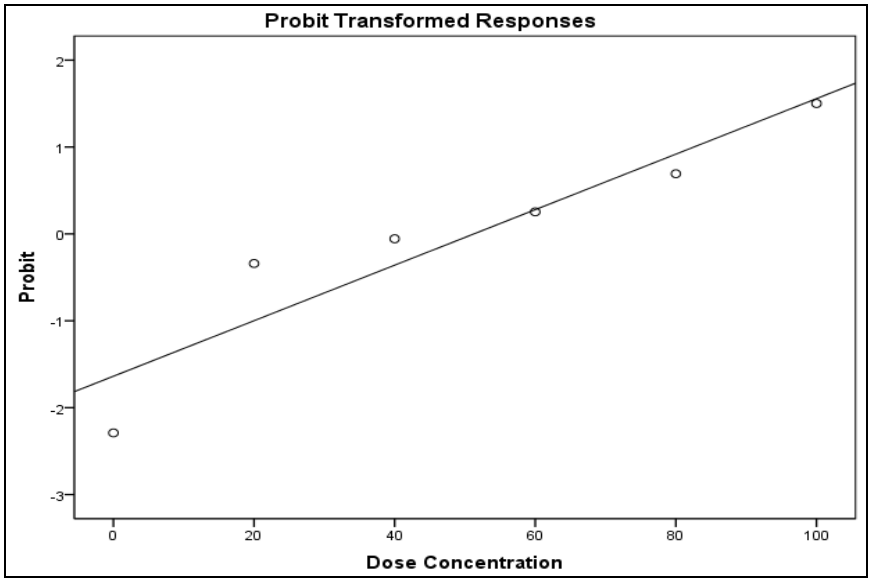

Fig 2: Probit transformed responses for concentrations v/s mortality against fourth instar larvae of *Anopheles annularis* (W.) treated with *Moringa oleifera* Lam. flowers extract after 48h

LC_{50} and LC_{90} of flowers extract was lowest i.e. 47.05 ppm and 95.61ppm against *Anopheles annularis*, on the other hand against *Anophels stephensi* it was reported to be 80.46 ppm and 130.14ppm respectively.

From the above mentioned statistical results it has been proved that *Anopheles annularis* is more susceptible towards *Moringa oleifera* flowers extract, exhibiting the lowest values of LC_{50} and LC_{90} on the other hand *Anopheles stephensi* is proved to be more resistant mosquito being a vector.

3.2 Polytene chromosome observation

In the present investigation efficacy of the plant extract used is calculated mainly based on dose mortality and potentiality on insect polytene chromosomes. We observed the changes in chromosome with live larva after the treatment and control experiments in two species of anopheline mosquitoes of which one is considered as vector and another is non vector. The normal salivary gland chromosomes complement of anopheline mosquito consist of three pairs of synapsed, banded, polytene chromosomes. The X-chromosome is the shortest and there are two longer autosomes, each with two arms, are present in each salivary gland cell. The bands (B) interbands (I) and chromocentre (C) are clearly visible in the preparations of control experiments of both the species (Fig 2 and 2).But in treated groups effects induced on polytene chromosome are clearly visible. These changes are especially structural in nature Fig. (a and b). Most frequent change observed was chromosome stickiness which was more frequently seen among the non vector species i.e. *Anopheles annularis*. Some chromosomes completely lost their basic structure like characteristic banding pattern and appeared clumped. Other changes include chromosomal break points in various arms and uneven lengths while other showed breakage of chromosomal arms. Thus it was also reported that due to the effects of flowers extract, polytene chromosome of *Anopheles annularis* become diffused or super contracted or shortened in greater intensity as compared to *Anopheles stephensi.* In other words the intensity of changes was reported to be more prominent in case of *Anopheles annularis* as compared to *Anopheles stephensi*.

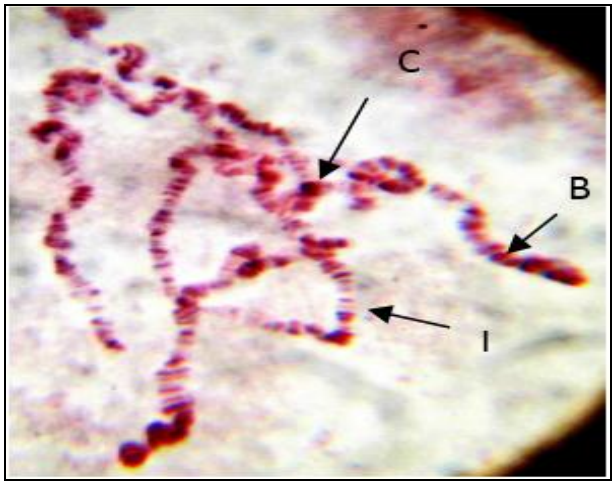

Fig. 2(A): Polytene chromosome of *Anopheles stephensi* mosquito larva (control), C-Centromere, B-Band, I-Interband

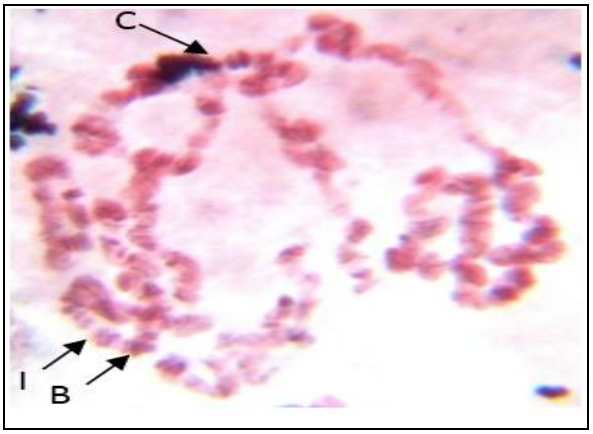

Fig 2(B): Polytene chromosome of*Anopheles annularis* mosquito larva (control), C-Centromere, B-Band, I-Interband

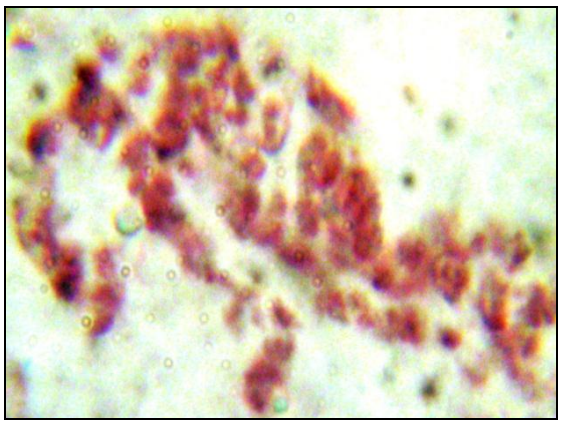

Fig 2(C): Polytene chromosome of *Anopheles stephensi* mosquito larva (treated)

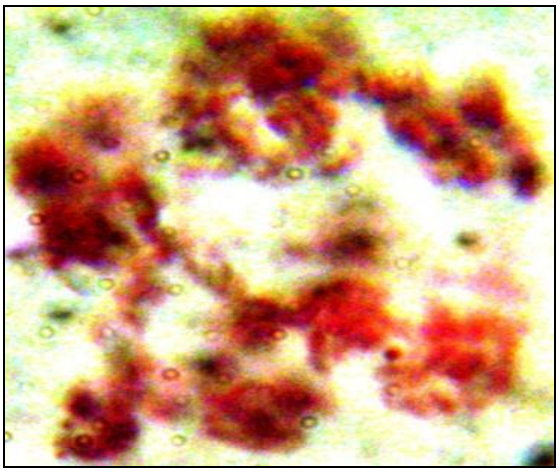

Fig 2(D): Polytene chromosome of *Anopheles annularis* mosquito larva (treated)

4. Discussion

A number of scientists identified, screened and isolated large number of chemical compounds from leaves and seeds of many botanicals for insect feeding avoidance and growth inhibition as toxicant [27-34]. From the educational point of view, plants were represented as a huge store house of effective and potential natural products and number of laboratories worldwide have examined thousands of species of higher plants for pharmaceuticals and as herbal biopesticides [35-40]. Zakaria [41] observed that compactness of chromosomes are depends on the efficacy of plant extracts is also observed in our study. The present work also confirmed the observations of the earlier studies [42] which showed that thio-TEPA is one of the best chemicals to induce chromosomal mutation in the mosquito *Culex P. fatigans.* The different types of chromosomal abnormalities noted in our study were exactly like to those of *Aedes aegypti* and *Aedes asbopictus* [42]. And the other abnormalities like the occurrence of intercalary and terminal breaks, chromosome stickiness, clumping of chromosomes, recorded in the present study had also been reported by Grover [43] in *Culex P. Fatigans* due to effect of Apholate, Metapa and Hempa independently.

The occurrence of stickiness, clumping, acentric and dicentric bridges and the formation of circular chromosomes in the present work had also been recorded by Rai [44], Tadano and Kitzmiller [45] using other experimental material.

5. Conclusion

In the present study *Moringa oleifera* Lam. flowers extract shows good effect on *Anopheles stephensi* and *Anopheles annularis* larvae and is also non toxic to human beings. The present observation also revealed that flowers extract of *Moringa oleifera* has a promising larvicidal efficacy. Plants are rich sources of bioactive organic chemicals and offer a promising advantage over synthetic pesticide as naturals are non toxic, less prone to development of resistance and easily biodegradable. The flowers extract of *Moringa oleifera* therefore plays an important role in the mosquito control. Dose mortality test observation showed that *Anopheles stephensi* mosquitoes are more resistant as compared to *Anopheles annularis* which are more susceptible and showed more pronounced effects of flowers extract on their polytene chromosome. Greater structural deformities and more clumping were observed in treated polytene chromosome of *Anopheles annularis*.

Secondly our present work on mosquito genetics not only improved our basic understanding of molecular biology, behaviour, vector genetics, physiology and roles in transmission but may also contributed to new strategies for controlling malaria. Thus the genetic study opens an exhilarating prospect to uncover the molecular mechanisms causative to vectorial capacity and to identify targets potentially helpful for vector management.

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