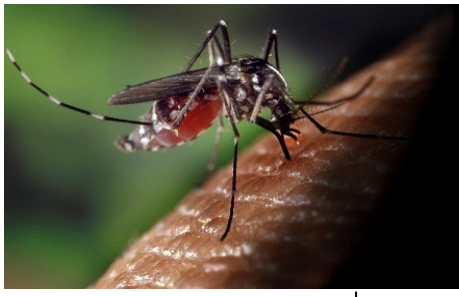

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Effects of plant leaves extract (*Moringa oleifera* Lam., *Catharanthus roseus* Linn., *Lantana camara* L. and *Thyme vulgaris* L.) on salivary gland chromosome of *Anopheles stephensi* Liston (Insecta: Diptera: Culicidae)

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Abstract
The use of natural plants products that reduce mosquito population can provide many associated benefits that limit pesticides environmental impact due to shorter latency and may be useful in preventing development of resistance. The larval mortality was observed at 24hrs and 48hrs of time exposure. LC_{50} value was calculated. Microscopic slides were prepared from live larvae and microscopic photography were done for the prepared slides and studied for visible changes. Dose mortality test observation showed the intensity of activity in a descending order as - *Moringa oleifera* Lam. > *Catharanthus roseus* Linn. > *Lantana camara* L.> *Thyme vulgaris* L. From this result we can conclude that more effective plant extracts results in more clumped/fused chromosomes. Effects of plant extract induced on polytene chromosome most frequently were structural in nature.

Keywords: *Anopheles stephensi*, plant extract, polytene chromosomes, salivary gland

1. Introduction
World Malaria is a devastating disease killing _1.5 million people every year, mostly among African children under the age of 5 malaria vectored by species of Anopheles mosquito remains one of the most prevalent mosquito borne diseases in the tropical world with 200-450 million infection up to 2.7 million deaths annually (WHO, 2010) [46]. Malaria threatens almost 1 to 3 million people every year. It is difficult to control mosquito borne diseases now due to lack of vaccines. WHO (2010) [46] 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 stephensi Liston (Diptera: Culicidae) is an important malaria vector in the Persian Gulf and South Iran (Manouchehri *et al*.1976) [27], in urban areas of the Indian subcontinent (Pant *et al*. 1981) [31], as well as in rural areas of North Pakistan and East Afghanistan (Rowland *et al*. 2002) [38]. This species is also an outstanding laboratory model system for malaria parasite transmission studies (Abraham *et al*. 2004) [2].
In the last 50 years, vectors have mainly been controlled with synthetic Insecticides. 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 (Omena *et al*. 2007) [30]. 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 (Govindrajan *et al*. 2008) [18].
Previous studies with various mosquito species have demonstrated that mosquito host feeding behaviours and vector competence to malaria parasites are under genetic control (Coluzzi *et al.* 1977; Collins *t al.* 1999) [10, 9]. Anopheline mosquitoes, like Drosophila, are renowned for the presence of polytene chromosomes (Coluzzi *et al.* 1979) [11]. These giant chromosomes were first described in larval chironomids by Balbiani (1881) [4] but only accorded proper significance 50 years later by Heitz & Bauer (1933) [20]
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 N. Rishikesh (1959) [37]. Moreira C.K, Bijovsky A.T. (1999) [29] A literature survey showed several reports on the study of the polytene chromosomes in *Culex quinquefasciatus* (Berger 1937, Kitzmiller 1954, Sharma *et al*. 1969, Kanda 1970, Patnaik *et al*. 1989) [5, 40, 25, 32]

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. Large number of plants has been evaluated in this respect. Moreover, insecticides from plant parts were measured as the suitable alternatives of chemical insecticides for its pest specific and biodegradable nature (Periera and wohlgemuth, 1982) [33].

The increasing interest to observe the effects of plant extracts at chromosome level which may lead to identify exact plants part for use as insecticides for other insects were the main objective of this investigation. There are number of publications with lists of plants which have insecticidal properties. Hence mosquito can be controlled by an alternative approach through the use of plant extracts. Natural insecticides is searched which do not possess any ill effects and which is easily degradable on non-target population.

2. Material and methods

2.1 Collection of plant

Mature leaves of *catharanthus roseus, Moringa oleifera, Lantana camara* and *Thyme vulgaris* plant were collected from nearby areas like Mohanlal Sukhadia University campus and other nearby regions of Udaipur (Raj.), India. After washing and drying the leaves in shade it was powdered in blender, filtered and the clear filtrate used for extraction without obtaining dry solids.

2.2 Extraction

After washing the leaves were shade-dried, and finely ground. The leaves powder (10g/solvent) was loaded in Soxhlet apparatus (Vogel 1978) [44]. The liquid were removed from the extract using a rotary vacuum evaporator to collect the crude extract. Standard stock solutions were prepared at 1% in methanol. From this stock solution, different ppm concentrations were made and were used for larvicidal bioassays.

2.3 Test organisms

Anopheles stephensi mosquitoes were reared in laboratory (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 per WHO protocol.

2.4 Test for mortility and biossay of larvae

The larvicidal activity of the methanolic plant extracts against the larvae *Anopheles stephensi* mosquito was determined by the method recommended by WHO (2005) [47]. 1% stoke solution of methanolic extract of each plant were prepared. From this stock solution different doses in ppm concentration were prepared like 20ppm, 40ppm, 60ppm, 80ppm and 100ppm. Thus the experimental group is the methanolic extracts of the leaves of *catharanthus roseus, Moringa oliefera, Lantana camara and Thyme with* 20ppm, 40 ppm, 60ppm, 80ppm and 100ppm concentrations. These concentrations were selected after the pre-test conducted. Batches of 30 forth-instars larvae of *Anopheles stephensi* 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 control mortalities were corrected by using Abbott's formula (Abbott 1925) [1]. The LC_{50} was calculated after 24 and 48 h by Probit analysis (Finney 1979) [15].

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

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 (2010) of "Methods in Anopheles research" which have explained the best methods for these techniques. The salivary glands of larvae were dissected in 5% propionic acid under dissecting microscope. The dissected glands were fixed in carnoys fixative followed by 50% propionic acid for three minutes until they have cleared and swollen to about twice their original size. Staining was done in 2% lacto aceto orcein for 4 minutes. The excess stain were removed with a piece of tightly rolled up absorbent paper and mounted in 50% propionic acid. 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. D.V. Jensen (1955) [23], Russel, P.F., West, L.S., and Manwell, R.D. (1946) [39] and Trembley, H.L. (1955) [42] have also cited good literature about it. Preparation of slides was done after the dissection of salivary gland from 4th instar larvae which remain alive after treatment and control.

3. Result

These studies have proved that number of plant species possess potentiality for pest controlling properties under laboratory conditions. In the present investigation the plant extracts of *catharanthus roseus, Moringa oleifera, lantana camara* and *Thyme vulgaris* were selected and biological activity of these plants were examined to find out its potentiality and effectiveness against mosquito larvae which can help in developing advance control strategies for these mosquitoes. Dose mortality assessment and tests for effect on chromosome have been done with the methanolic extract obtained from soxhlet apparatus. The data of all the four plants were analyzed and the results were illustrated in Table as below:

Table 1: Toxicity of leaves extract of *Catharanthus roseus, Moringa oleifera, Thyme Vulgaris* and *Lantana camara against Anopheles stephensi* (L) under 24h and 48 h exposure time

Plant leaves extract	LC_{50} value in 24 hr	LC_{50} value in 48 hr
1.*Catharanthus roseus*	24.81	21.12
2.*Moringa oleifera*	39.98	28.25
3. *Thyme vulgaris*	48.67	40.45
4. *Lantana camara*	61.88	53.34

3.1 Dose-mortality assessment

The dose mortality results of methanolic plant leaf extracts against larvae of Anopheles were found promising. These extracts had been found strongly effective against the 4th instar larvae of *Anophelis stephensi* mosquito. After carefully counting the live and dead larvae in experimental and control group its percentage mortality were calculated. The LC_{50} value for *C. roseus* extract was 24.8 ppm for 24h and 21.12 ppm for 48h experiments. The LC_{50} value for *M. oleifera* extract was 39.98 for 24h and 28.25 for 48h exposures. The LC_{50} value for *T. vulgaris* extracts was48.67 ppm for 24h and 40.45ppm for48h of exposures. Similarly the LC_{50} value for *L. camara* was 61.88 for 24h and 53.34 for 48h experiments. According to the effectivities of the plant extracts against 4th instar larvae the plant could be arranged in a descending order as *Catharanthus roseus* > *Moringa oleifera* >*Thyme vulgaris* > *Lantana camara* with remarkable difference in structure of chromosome. However, in the present investigation efficiency of the plants used is calculated mainly based on dose mortality and potentiality on insect polytene chromosomes.

3.2 Salivary gland chromosome observation

In this study we observed the changes in chromosome with live larva after the treatment and control experiments. The normal salivary gland chromosomes of *A. stephensi* 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) were clearly visible in the preparation (Fig.1).But in experimental groups effects of plant extract induced on polytene chromosome are clearly visible. These are both physiological and structural in nature (Fig.2). Most frequent change observed was chromosome stickiness. Regarding physiological change chromosomes completely lost their basic structure like characteristic banding pattern and appeared clumped. Regarding the structural changes some chromosomes showed break points and uneven lengths while other showed breakage of chromosomal arms. The effects of plant extract were also results in the breakage and fusion of the chromosomes. In some cases the broken pieces of chromosome were stayed separately. Thus we observed that highly effective plant extracts showed more compact chromosomes than lower effective one.

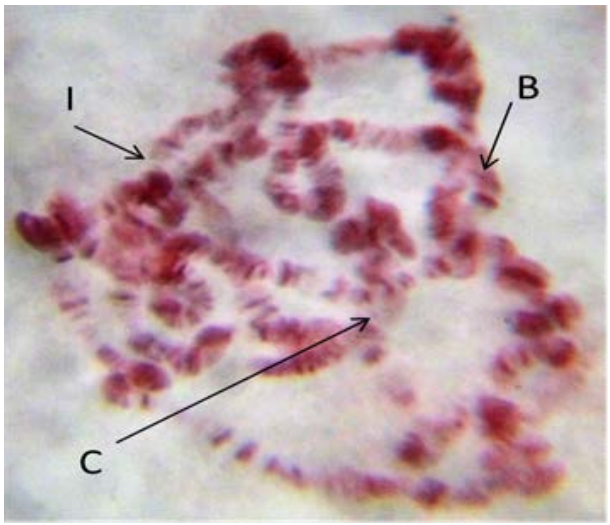

Fig 1: Polytene chromosome of *Anopheles stephensi* mosquito (control)

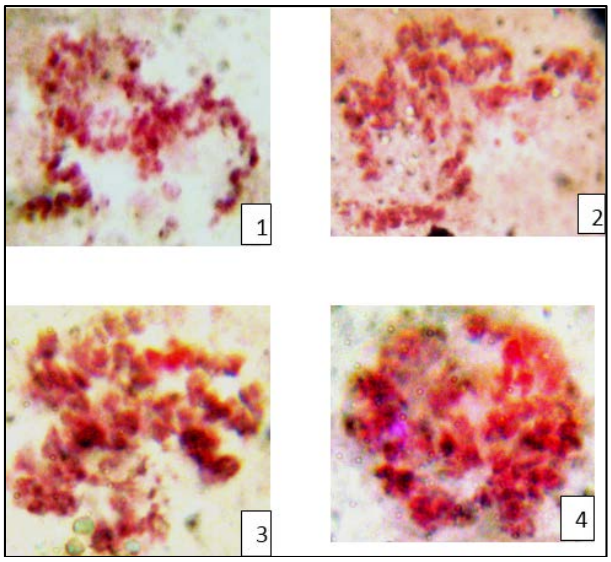

Fig 2: Effects of plant extracts on polytene chromosome 1-Effects of *Lantana camara* 2-Effects of *Thyme vulgaris* 3-Effects of *Catharanthus roseus* 4-Effects of *Moringa oliefera*

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 (Jacobson *et al*., 1975; Bernays and Chapman, 1977; Doskotch *et al*., 1977; Carpenter *et al*., 1979; Warthen, 1979; Jurd and Manners, 1980; Menn, 1980; Ho *et al*., 1995) [22, 6, 14, 8, 45, 24, 28, 21]. From the educational point of view, plants were represented as a huge storehouse 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 (Van Beek and Breteler, 1993; Gonzales-Coloma *et al*., 1994a, b; Addor, 1995; Cornelius *et al*., 1995; Blaske and Hertel, 2001) [43, 16, 17, 3, 13, 7]. The compactness of chromosomes depend on the efficacy of plant extracts (Zakaria *et al*., 2012) [49] as also observed in our study. The present work also confirmed the observations of the earlier studies (Puttaraju, 1988) [34, 35] 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* (Puttaraju, 1988) [34, 35]. 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 *et al*., (1973) [19] 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 (1963) [36], Tadano and Kitzmiller (1969) [41] and Grover *et al*., (1973) [19] using other experimental material.

5. Conclusion

Thus it can be concluded that plant extract effects salivary gland chromosome as a whole and hence disrupt the genetic material which result in the death of mosquito larvae. This means these herbal bio pesticide contain biodegradable properties which destroyed the chromosome and cause death of larvae. These important findings lead us for studying

chromosomal changes due to the effects of plant extracts. From this result we can conclude that more effective plant extracts results in more clumped/fused chromosomes. Thus the present study proved that the plant extracts have some physiochemical properties which effected directly to the chromosomes and play an important role in killing Anopheles larvae. To conclude the results obtained in the present study open the possibility for further investigations of the efficacy of natural product extracts as a potential agent for combating mosquitoes.

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