RESEARCH ARTICLE

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Corresponding Author
Lovleen
Mosquito Cytogenetics Unit,
Department of Zoology,
Panjab University Chandigarh-160014
India
Email:
lovleenmarwaha@gmail.com

QR Code for Mobile users

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In vivo mutagenicity assessment of ethion pesticide using polytene chromosomes of *Anopheles culicifacies* *(Diptera: Culicidae)*

Lovleen
Mosquito Cytogenetics Unit, Department of Zoology,
Panjab University Chandigarh-160014
India

ABSTRACT
Excessive use of synthetic pesticide formulations has become an indispensable requirement in modern agricultural executions, to fulfill growing demands of food for continuously increasing human population. Consequently, the genotoxicity evaluation of corresponding xenobiotics has become crucial prerequisite, to assess their drastic and substantive effects on the integrity of genetic imprints of exposed organisms. The present investigation is attributed to the genotoxicity evaluation of ethion, employing hereditary material of *Anopheles culicifacies* exposed to sublethal aliquot, LC_{20} for continuation of 24 hours, implementing an *in vivo* approach *viz*: polytene chromosome aberration analysis. Exposure of concerned pesticide, ethion was given to second instar larvae, subsequently, larvae were reared upto fourth instar stage which were sacrificed for the study of salivary gland polytene chromosomes. Observations indicated that ethion elevated percentage frequency of discrepant aberrations, eventually expressed through structural aberrations such as ectopic pairings, inversions, chromosomal fusions, translocations, asynapses and breaks with statistical corresponding 27.35±9.49 in ethion treated stocks as compared 19.36±4.63 in control groups. Statistical analysis indicated that concerned organophosphate pesticide induced significant genotoxicity at sublethal dose.

Keywords: *Anopheles culicifacies*, ethion, polytene chromosomes

INTRODUCTION

Reduction of agricultural areas due to urbanization, industrialization and unsustainable land erosion, has consequence an apparently challenging dilemma of adequate supply of food to continuously growing human population, world widely. Consequently, for overwhelmingly production of agricultural commodities, tons of synthetic pesticides are being inevitably sprayed on food crops that besides causing detrimental impacts to target species, also have drastic consequences on nontarget species including human beings[1]. The most adverse effects of indiscriminate applications of such synthetic chemicals is their long term persistence in environment and specific bio-accumulation properties in living organisms, resulted many negative health hazards on society in general and vulnerable population like children in particular. Numerous reports are procurable in scientific literature, attributed to the acute and chronic impact of these synthetic formulations, whose excessive implementation has resulted in genetic damages, chronic neurodegenerative disorders, reproductive and developmental abnormalities [2-9]. Although, with implication of *in vitro* and *in vivo* conventional technologies, using appropriate biomarkers, the genotoxic properties of these synthetic chemicals are well documented but it is not possible for many countries to reduce the use of such compounds without reducing crop yields[10]. Consequently, their genotoxic evaluation has become crucial aspect that should be conducted out on intensive levels so that specific recommended exposure limit of these formulations can be concluded in such a way that predicted concentration for application, should be lethal to target pests but should not have any destructive consequence neither to other species of animals nor to environment.

The objective of the present study was determination of cytotoxicity and clastogenic properties of a commercial organophosphate pesticide, ethion, at sublethal dose by using *Anopheles culicifacies* genome. Ethion (O,O,O',O'-Tetraethyl S, S'- methylene bis - phosphorodithioate), is a member of organo-thiophosphate family of the organophosphate insecticides, moderately persistent, nonsystemic concomitantly broad spectrum insecticide, registered for commercial purposes in 1965. Nowadays it is extensively employed to regulate mites, leafhoppers, maggots and foliar feeding larvae on a wide variety of food, fiber and ornamental crops; additionally, concerned pesticide is practiced on greenhouse crops, lawn, fiber and ornamental crops. Ethion is highly to moderately toxic compound, persistent continuation, in a particular environment depends upon climatic conditions; half-life period of ethion is

1.3 to 8 weeks, whereas, EPA, on the basis of toxicity of the concerned compound, designated it, as class II formulation. Mode of action of ethion is to inhibit acetylcholinesterase enzyme (AChE) which is required for breaking acetylcholine neurotransmitter molecules into choline and acetate group. Blockage of AChE, results in accumulation of acetylcholine neurotransmitter at the synaptic junction, eventually prompting continuous stimulation of nerve impulses, consequently, resulting hyperexcitation, voluntary muscles become twitched, and ultimately the insects are killed.

Currently, a variety of biomarkers are used to assess the potential adverse effects of different environmental mutagens on genetic imprints and reproduction potential of vast variety of experimental models. Correspondingly, mosquito genome has proved quite efficient analytical material for genotoxicity assessment of various environmental contaminants and has been widely used in various evaluation researches. In such studies, induced damages to genetic material have been measured in term of increased percentage frequency of chromosomal aberrations[11-15]. *Anopheles culicifacies* was selected as test organism for present investigation due to its easy availability in nature, adaptability to laboratory conditions, short life span and presence of giant sized polytene chromosomes. Larval salivary polytene chromosomes of various *Anopheline* species has been used as analytical tool in many studies, attributed to mutagenicity evaluation of various chemical agents like antitumour and anticancerous drugs, aromatic amines, mitostatic drugs and salts of various heavy metals like nickel, mercury and lead[16-17]. The deleterious effects of these compounds can be observed in the form of various types of aberrations such as inversions, translocations, breaks, deletions and asynapses of somatically paired homologues of polytene chromosomes. Considering all that facts salivary gland polytene chromosomes of *Anopheles culicifacies* were considered as an assessing tool for present genotoxicity evaluation study.

Although, numerous *in vitro* and *in vivo* investigation are available in literature review, which provide insight into a variety of chronic genotoxic properties and other deleterious effects of various pesticides on different test models[18-21] . But only limited studies have been carried out on evaluation of clastogenic and cytotoxic effects of ethion on genetic material, hence motivate by this reason, present research has been executed. The present *in vivo* polytene CA assessment studies has been designed to investigate the correlation between inductions of increased percentage frequency of various aberrant chromosomal configurations in salivary chromosomes, with exposure to ethion, in comparison to natural population. During present investigation the induced percentage frequency of various structural aberrations were compared with that of control and statistically level of significance was analyzed by application of student 't' test.

MATERIALS AND METHODS

Pesticide tested— Ethion (IUPAC name O,O,O',O' – Tetraethyl S,S' methylene bis (phosphorodithioate), CAS number 563-12-2, molecular formula $C_9H_{22}O_4P_2S_4$ and molecular weight 384.5), belongs to organothiophosphate family of organophosphate insecticide which is commercially used to control various types of pests on different crops. Its circumstantial chemical structure is elucidated in Fig.1. For execution of present exploration, a packet of 100 ml available under trade name ethion (M/S Krishi Rasayan Exports Ltd, India) was procured from market, and was used as such because the basic motive of present investigation was an evaluation of clastogenic properties of this commercial formulation, which is really going to field. Many investigations, dealing with pesticides genotoxicity evaluation, indicated difference between active as well as inert ingredient with respect to mutagenicity, furthermore main conclusion from such investigations also showed that inert ingredients are more toxicity than mutagenic so such compounds generally increase toxicity of synthetic formulations.

Experimental test organism— *Anopheles culicifacies* was selected for present investigation as it provides good quality temporary squash preparations of salivary polytene chromosomes. *Anopheles culicifacies* is a principal vector of malaria in rural and peri-urban areas of India [22]. The adults of the species prefer to rest indoors in cattle sheds and human dwellings. To achieve the target, the gravid females of this species were collected from a village Nada sahib, about 20 km southeast of Chandigarh, with the help of mouth aspirator and were carried to laboratory in small field collection cage made of mosquito net cloth. Subsequently, those captured gravid female mosquitoes were individually transferring to different test tubes, identified by examined with help of 5X magnification hand lens, by following suitable identification key [23]. After that those gravid females were allowed to oviposit in water filled petridishes already placed in mosquito breeding cages at 25 ± 1ºC, 70 ± 10% humidity and 12h/12h photoperiods[24-26]. Egg rafts obtained from them were allowed to hatch in BOD incubator set at optimal conditions of temperature and humidity. The larvae hatched from them were fed on protein rich diet made from dog biscuits and yeast extract mixed in ratio 6:4,

quantity of feed was adjusted according to density and stages of larvae. The rearing medium was changed daily in order to avoid scum formations which interfere with respiration of larvae.

Standardization of dose and mode of exposure—LD_{20} was calculated on the basis of mortality of second instar larvae exposed to serial dilution concentrations of stock solution of pesticide (1%) for 24 hours. Desired concentrations of serial dilution were prepared by adding aliquots of the stock solutions in distilled water. To test each of these concentrations, three replicates of twenty larvae were kept simultaneously, with respective controls under controlled conditions of laboratory. The mortality of larvae was monitored after 24 hours and only larvae whose bodies were completely devoid of the exuviae were recorded as alive. The exact LD_{20} value was calculated by applying probit analysis which was 56.23 nl/ml for ethion during present research [27] (Fig. 2). The mortality in the control group was taken to be the natural response rate. For polytene chromosomal study, second instar larvae of *Anopheles culicifacies* were given treatment of statistically analyzed LD_{20} for 24 hours, subsequently; they were transferred to distilled water and were reared by feeding on protein rich diet.

Slide preparation and statistical analysis: Fourth instar larvae were sacrificed for making temporary squash preparation by following standard staining procedure of French *et al*., 1962 with suitable modifications such as dilution of stain and duration of staining [28]. Slides were screened under digital phase contrast microscope and various chromosomal complements with proper spreading and staining were photographed. Those compliments were studied for various types of structural chromosomal aberrations such as inversions, translocations, deletions, ectopic pairings, asynapses, chromosomal fusions and breaks. From obtained data for ethion treated stocks and controls, the percentage frequency of various type of aberrations were determined, subsequently, procured data was statistically analyzed.

RESULTS

Polytene chromosomal aberrations analysis—In polytene chromosome aberrations study, the genotoxicity of ethion was observed in the form of aberrant mitotic configuration represented mainly in the form of structure chromosomal damages such as ectopic pairing, inversions, chromosomal fusion, translocations, asynapses and breaks. During the present course of research work, a total of 58 different types of aberrations in the form of 13 inversions, 30 ectopic pairings, 1 asynapsis, 13 chromosomal fusions and 1 break were observed in the polytene chromosomes of nontreated larvae with percentage frequencies 12.75, 29.41, 0.98, 12.75 and 0.98 respectively. The incidence of such aberrations was significantly higher in the ethion treated stocks as there were as many as 82 aberrations which included 27 inversions , 5 translocations , 31 cases of ectopic pairings, 3 regions with asynapsis of synapsed homologues, 13 chromosomal fusions and 2 breaks. The percentage frequency of these aberrations was 26.47, 4.9, 30.39, 2.94, 12.75 and 1.96 respectively. In summary, the frequency of various CA showed significant difference between chemically exposed groups and control groups. Moreover, frequency of normal cells decreased as the frequency of aberrant cells with dislocated chromosomes increased during treatment. All this obtained data is specifically illustrated in Figure 4. Statistical analysis of this data corresponds to mean percentage (±SD) of the aberrant chromosomes was significantly higher in treated groups than controls, as indicated by the values 27.35±9.49 as compared to 19.36 ±4.63 in control groups (Table 1). Furthermore, when the obtained values were analyzed statistically, through student't' test, the apparent discrepancy between treated and control were found to be highly significant ($p<0.05$).

Nature of stock	Inversion Mean±S.D.	Translocation Mean±S.D.	Ectopic pairing Mean±S.D.	Asynapsis Mean±S.D.	Fusion Mean±S.D.	Break Mean±S.D.	Total Mean±S.D.	't' value
Control	4.34±0.58	-----	10.00±1.73	0.34±0.58	4.34±1.16	0.34±0.58	19.36 ±4.63	3.55
Treated	9±1.00	1.67±1.53	10.34±2.88	1.00±1.00	4.34±2.08	1.00±1.00	27.35±9.49	

d.f. = 4
$p < 0.05$
(S.D.=Standard Deviation, d.f.= degree of freedom)

Table 1. Statistical analysis of abeerations in the control and ethion treated stocks in polytene chromosomes of *Anopheles culicifacies.*

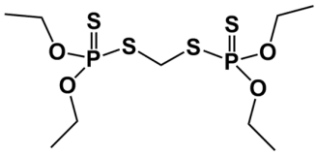

Figure 1. Chemical structure of ethion [43]

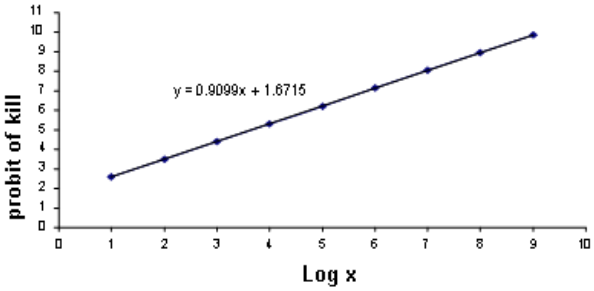

Figure 2. Relationship between the probit of kill and dose of ethion for *Anopheles culicifacies*.

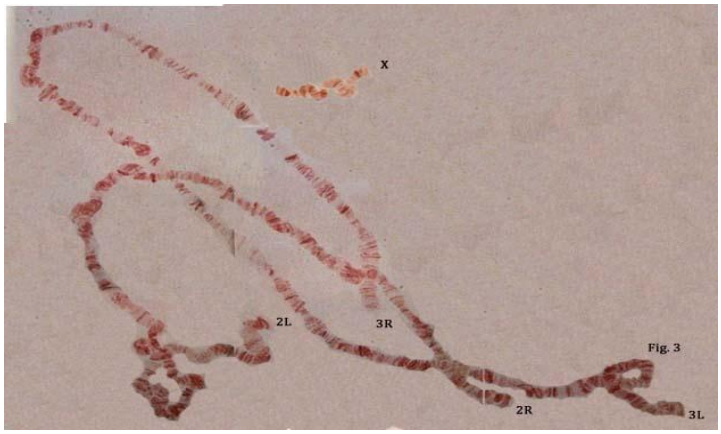

Fig 3. Normal compliment of polytene chromosome obtained from control stock of *Anopheles culicifacies.*

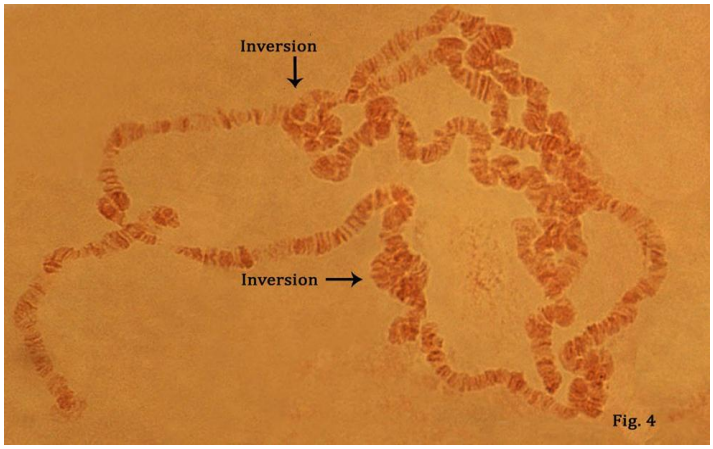

Fig 4: Paracentric heterozygous inversions in ethion treated stock.

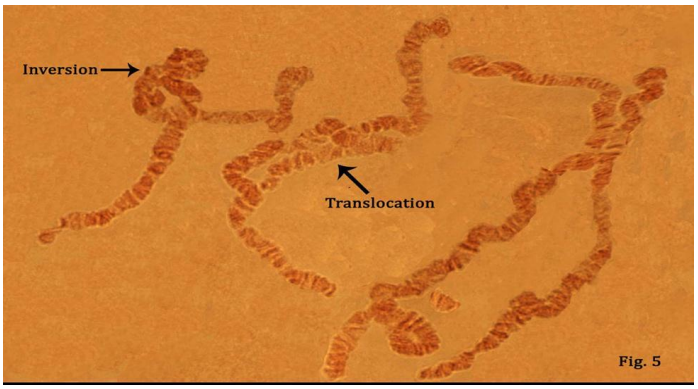

Fig 5: Paracentric heterozygous inversions and translocation in ethion treated stock.

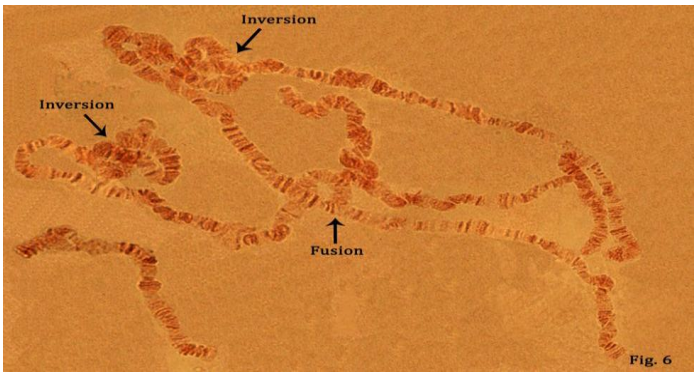

Fig 6: Paracentric heterozygous inversions and translocation in ethion treated stock.

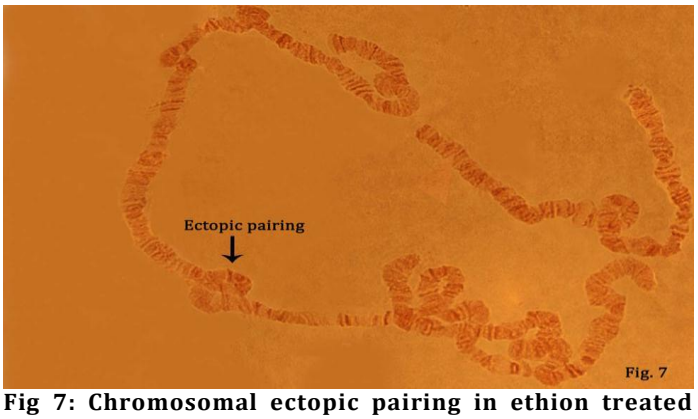

Fig 7: Chromosomal ectopic pairing in ethion treated stock.

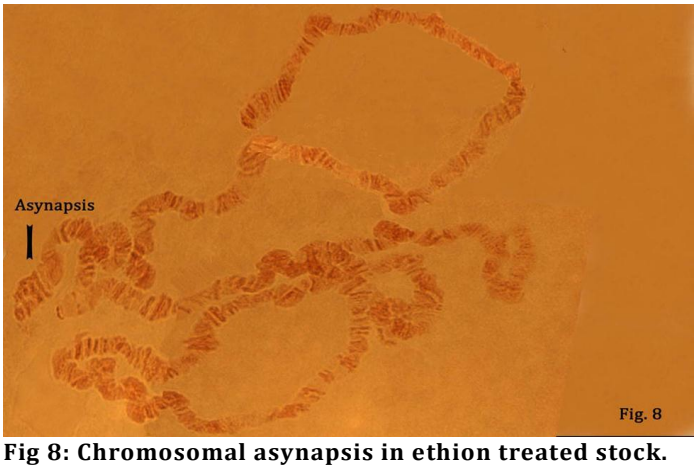

Fig 8: Chromosomal asynapsis in ethion treated stock.

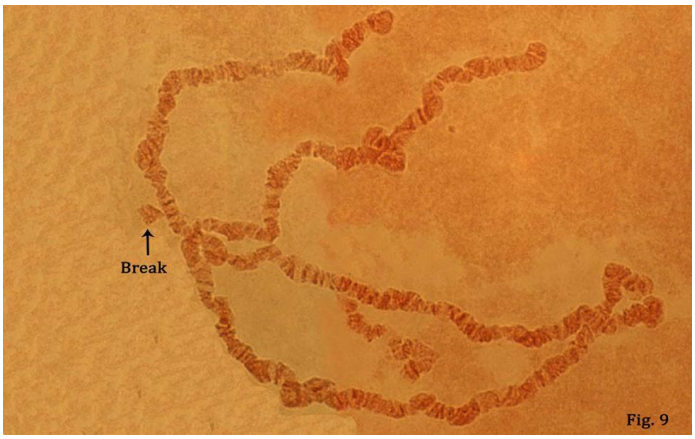

Fig 9: Chromosomal break in ethion treated stock.

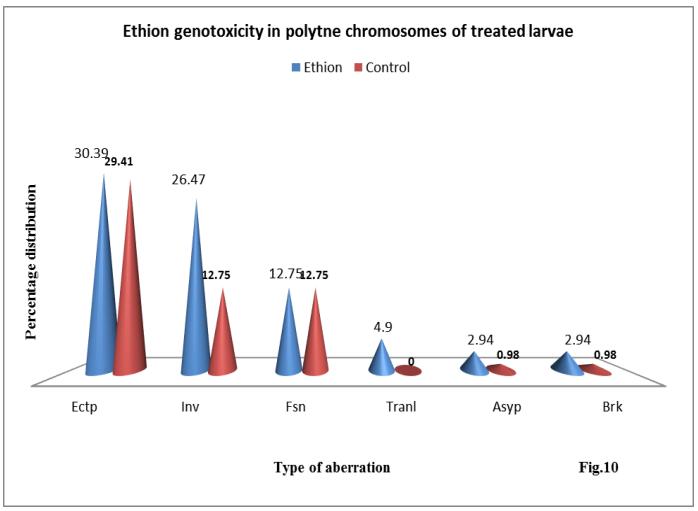

Fig 10. Percentage distribution of chromosoal aberrations in polytene chromosomes of *Anopheles culicifacies* treated with ethion. Maximum aberrations were in form of chromosomal ectopic pairing while minimum number was for chromosomal asynapsis and break. Asynapsis and chromatid breaks were observed in equal percentage frequency (Ectp-Ectopic pairing, Inv-Inversion, Fsn-Fusion, Asyn-Asynapsis, Tran-Translocation, Brk-Break).

DISCUSSION:

Normal metaphasic karyotype of *Anopheles culicifacies* present six unequal homologous pairs comprising a pair of sex chromosome (XX-female, XY-male) and two pairs of autosome out of that one pair is metacentric while other is submetacentric (Fig.3). During polytene chromosome formation, two homologous get synapsed with each other and subsequently undergo multiple round of endoreduplication. Therefore, salivary polytene chromosome preparations from the healthy and active fourth instar larvae present a set of three unequal and well banded elements comprising of a short X-chromosome and two much longer autosomes of unequal size. In majority of the preparations, all the three polytene chromosomes found connected by their centromeric positions to form a heterochromatic mass called chromocenter .They are the right and left arms of chromosomes 2 and 3 (2R, 2L, 3R, 3L) and a long arm of the X-chromosome. Due to multistranded nature of polytene chromosomes, any structural aberration is usually expressed in easy dateable form. During, present research exploration various types of chromosomal aberrations observed are inversions (Fig. 4), translocations (Fig. 5), chromosomal fusions (Fig. 6), ectopic pairings (Fig. 7), asynapses (Fig. 8) and breaks (Fig. 9). Comparison, of percentage frequency indicated astoundingly elevation in incidences of structural chromosomal aberrations in salivary polytene chromosomes of treated stocks than control groups (Fig.10) which apparently demonstrated the association of induced genotoxicity with exposure of pesticide ethion. It was also observed that in genetic material of treated individuals, ethion has maximum tendency to cause structural CA inform of chromosomal ectopic pairings concurrently with heterozygous inverted regions while lowest frequency of aberrant cells with chromosomal break and asynapsis were discerned from pesticide exposed population. After ectopic pairings, chromosomal inversions were detected as most frequent aberrations as compared to other chromosomal abnormalities. Characteristic chromosomal abnormality mainly represented in the form of translocation was only present in salivary polytene chromosomes pesticide exposed groups, while not even a single incidence of such chromosomal deformity has been observed in control stocks.

Procured data from polytene chromosomal aberration study indicated that ethion caused maximum incidences of ectopic pairings. Research investigation attributed to genotoxicity evaluation of various mutagens suggest that such type of chromosomal deformity generally occur due to increase in interspersed repetitive DNA content[28-29] . Therefore, elevated incidence of ectopic pairing in treated stocks, could be resulted due to interaction of ethion with genetic material. Furthermore, percentage frequency of chromosomal inversion also was enhanced in treated stock. Numerous explorations indicted that different environmental mutagens such as drugs, preservatives, pesticides and radiations caused chromosomal inversion in the genetic material by inducing breaks in it at certain weak points [30-32] , it suggest that ethion elevated the incidences of interstitial breaks during its interaction with genetic material of treated stocks.

Elevated incidence of chromosomal fusions were encountered during the course of present investigations and according to an investigation, this type of chromosomal change arose due to chromosomal stickiness and breakage [33]. A hypothesis consistent with this claim states that chromosome stickiness results from changes in specific non-histone proteins such as topoisomerase II and the peripheral proteins which form integral components of the chromosomes. Furthermore, another study concluded that fusion between chromatids could also be initiated by the simultaneous breakage of two chromatids or by the loss of telomere capping[34] whereas, an

investigation on *Drosophila* indicated the role of certain proteins in the process of chromosomal fusions which helped in the repair of telomeric breaks, arose after exposure to mutagens[35]. High incidences of chromosomal fusion in treated stocks, could result from increased stickiness in chromosomes due to genotoxic effects of concerned pesticide.

As compared to the elevated frequency of inversions, fusions and ectopic pairings, pesticides treated individuals suffered from comparatively lower frequency of chromosomal translocations, asynapsis and breaks. Literature review suggest different reasons for all these chromosomal aberrations : a study of spermatogonial stem cells of mice detected an elevated incidence of chromosomal translocations, after exposure of X-rays on mice and concluded that chromosomal translocations were proportional to DNA content present in those regions of the chromosomes which suffered such errors[36] whereas, another investigation in *S. cerevisiae*, indicated that the breakpoints of the translocations were usually flanked by direct repeats of 2–20 base pairs[37]. Furthermore, conclusion from a cytological study of leukemia in human subjects, specified the exact mechanism involved in the induction of chromosomal translocations[38] , according to which various types of genotoxic stress factors get bound to androgen receptors (AR) which promote site-specific DNA double-stranded breaks (DSBs) leading to translocations. For asynapsis : studies carried out so far, relate asynapsis aberration to the denaturing of the binding proteins due to the action of chemicals. An exploration indicated the incidents of asynapsis were more in polytene chromosomes of mosquito larvae treated with lead and mercury than in their control stocks [39].

Beside this, few exploration procurable in scientific literature based on different *in vivo* and *in vitro* studies about mutagenicity of ethion documented the genotoxic instinct of this formulation. Concerned synthetic formulation was found to cause various types of chromosomal aberrations in chick bone marrow cells[40] while a study in *Allium cepa* root tip cells indicated that synthetic compound has potential genotoxic effects on mitotic division as it induced scattered prophase, non-synchronized condensation of chromosome, disturbed prophase, equatorial plate shifting, sticky chromosomes, C-metaphase and sticky metaphase which was due to the interference of ethion in the normal process of mitosis in exposed cells[41]. Ethion was found to reduced RNA/DNA ratio by reducing synthesis of nucleic acid content and decrease in the protein content by reducing their synthesis in *Bombyx mori* [42]. Outcomes of present research exploration also support the cytotoxic and clastrogenic instinct of ethion to genome of *Anopheles culicifacies.*

CONCLUSION:

Ethion elevated percentage frequency of various structural aberrations such as ectopic pairing, inversions, chromosomal fusion, translocations, asynapses and breaks which could be resulted due to interaction of concerned formulation with genetic material. Results from present exploration suggest that ethion at LD_{20} exposure limit, has induced statistically significant genotoxicity on polytene chromosomes of *Anopheles culicifacies*. Moreover extensive efforts are required for further genotoxic evaluation of these pesticides on more intensive level before using such formulations on wider scale. As such these agrochemicals although are quite beneficial but due to their considerable genotoxicity, the non-chemical alternatives include cultural practices, use of biological products and agents including beneficial insects which are natural enemies of target pests should be encouraged . The use of such natural alternatives should be enhanced by providing appropriate technology that allows for mechanized application on a wider level.

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