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GENOTOXICITY ASSESSMENT USING CHROMOSOMAL ABERRATION TEST IN FISH *CYPRINUS CARPIO* FOLLOWING SUB-LETHAL EXPOSURE TO KARANJIN.

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ABSTRACT
Much literature is available on the genotoxicity caused by synthetic pesticides. Very few literature is available on the genotoxicity – chromosomal aberrations in fish species exposed to botanical pesticides. Hence the aim of the present study is to find out if chromosomal aberrations occur in *Cyprinus carpio* following sub-lethal exposure to Karanjin. The changes caused by various kinds of toxic substances and pollutants at the genetic level can be detected in fish at specific level by using various genotoxic tests. The best method to study the effect of pollutants and toxicants at the level of chromosomes in fish is by the chromosome aberration test (CAT) in tissues kidney or blood. The chromosome aberration test is very easy, affordable and gives best results. The chromosomal aberration test in kidney and blood was performed by standardised method. After the completion of sub-lethal exposure period fish were collected from both control and exposed groups, kidney and blood were collected. The tissues were homogenised, fixed, centrifuged and well spread chromosomes were observed for different kinds of aberrations. Significant chromosomal aberrations were recorded in tissues of fish - *C. carpio* following sub-lethal exposure to Karanjin. Different types of chromosomal aberrations observed in the present study were Chromatid gaps, centromeric gaps, chromatid separations, ring chromosomes, short chromosomes, pulverisation and highly distorted chromosomes. The findings of the present study clearly indicate that Karanjin though a furano-flavonoid may cause considerable genotoxicity in non-target aquatic organism especially food fish, if present at very low concentration for a long duration and may also affect the overall health of fish. Hence the use of any kind of substances in agriculture and aquaculture field, whether obtained as plant secondary metabolites or from synthetic origin, should be used carefully and under proper guidance.

KEYWORDS: Blood; chromosomal aberrations; *Cyprinus carpio*; Flavonoid; Genotoxicity; Karanjin; kidney.

INTRODUCTION

Plants have been proven to have structurally-diverse and biologically active substances having medicinal and pesticidal properties. According to the studies conducted by Istvan (2000) plants are considered to be an inexhaustible source of many important secondary metabolites. Since many ages the importance of neem, it's medicinal and pesticidal importance to mankind is well established by WHO (2003). The active ingredient isolated from the seed kernels of Neem is Azardirachtin, it has been used extensively in the manufacture of many pesticides with different concentrations and different brand names. We assume that these active metabolites as they are synthesised in and isolated from plants, are very safe and do not have any impact on non-target organisms. It was recently found that a neem-based pesticide - Achook, showed toxicity to zebrafish - *Danio rerio* by Ansari and Sharma (2009). There are thousands more trees possessing active metabolites with pesticidal properties. One of the plant species recently gained attention and interests of researchers and scientists are *Pongamia pinnata* also known as *Derris indica*, commonly known as karanj plant. The most important active metabolite isolated from the seeds of this plant is a flavonoid called Karanjin. Cytotoxic nature of various parts of leguminous plants have been reported by the experiments conducted by Khalighi-Sigaroodi *et al*. (2012). This active ingredient is being used in the manufacturing of bio-pesticides and bio-insecticides in different names, one of them being Derisom was reported by Tamrakar *et al*. (2008); Al-Muqarrabun *et al*. (2013).

Pollutants, toxicants and pesticides in aquatic environment induce several chromosomal abnormalities in the aquatic organisms. It has been reported by some researchers that some of the pollutants when present for long durations and in less concentrations cause considerable degree of chromosomal aberrations in aquatic organisms especially fish was suggested by Barker and Rackham (1979). Chromosomal aberration test in recent years have received considerable attention as a biomarker technique in the evaluation of genotoxicity of various toxicants present in the environment was

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reported by Dar *et al*. (2016). The most common types of aberrations seen in fish are categorised as chromosome breaks, ring chromosomes, dicentric chromosomes, chromatid gaps, chromatid breaks, chromatid exchange, stickiness, fragments, deletions, centromeric gap, attenuation, stubbed arms and polyploidy. Different pesticides affect the cells at different stages of cell cycle. Several authors such as Das and Nanda (1986); Al-Sabti and Metcalfe (1995); Kushwaha *et al*. (2003) have reported genotoxic effects of different kind of pollutants in different species of fish using cytogenetic analysis. An advantage of using cytogenetic studies is that it reveals a measure of sub-lethal effects of xenobiotics in various organisms. So far no studies have been conducted regarding toxicity of Karanjin and also genotoxicity studies related to Karanjin in *Cyprinus carpio* or any other freshwater fish species. Hence, the aim of the present study is to study chromosomal aberrations in *Cyprinus carpio* following sub-lethal exposure to Karanjin.

MATERIALS AND METHODS

Procurement maintenance and treatment of fish: Juveniles of *Cyprinus carpio* ranging in length 14 to 16 ± 1.35 cm and weighing 38.16 to 40.25 ± 2.57 gm were procured from an aquaculture pond located in a village in Kaikaluru district of Andhra Pradesh state. Fishes were maintained at the Department Animal House Facility in well aerated tanks for a period of one month. Care was taken to avoid overcrowding. Fish were fed twice daily with commercially available fish feed pellets (Tayo grow) and the water was renewed as and when required. The 96 hrs LC50 value of Karanjin (Derisom) was previously estimated as 2.8 ppm. $1/10^{th}$ of the 96 hrs LC50 value i.e., 0.28ppm was taken as the sub-lethal concentration. Fish were exposed to the sub-lethal concentration of Karanjin for a period of 21 days in a semi static method. After the completion of 24 hrs, 7 days, 14 days and 21 days kidney and blood were collected from both control and exposed group fishes and were used for the preparation of chromosomes to study the different types of aberrations. Kidney and blood are selected to carry out the chromosomal aberration studies as they provide better chromosome spreads. The experiment was performed in six replicates.

Details about Karanjin: In general Karanjin is Furano-flavonoid isolated from the seeds of *Pongamia pinnata* also known as *Derris indica.* Karanjin has been proven to have pesticidal properties by Pavela (2009); Majumdar (2002); Majumdar *et al*. (2004); Tamarakar *et al*. (2008); Al-Muqarrabun *et al.* (2013). The source of Karanjin in the present study is Derisom, which is a biopesticide available in liquid formulation. Acute toxicity or 96hrs LC50 value of Karanjin based biopesticide Derisom to the common carp – *Cyprinus carpio* was already determined by Finney's probit analysis method (1971).

Preparation of Chromosomes from Kidney and Blood: Chromosomal aberrations were assessed by the method of Nagpure *et al*. (2007) with some modifications. After the completion of exposure period, the fishes were injected with 0.05% Colcimide as given by OSPAR (2002) intramuscularly @ 1 ml per 100g of body weight. Fishes were kept alive for 1.5-2 hours in well aerated containers. Fishes were anaesthetized with ethylene glycol, kidney was dissected out and cut into small pieces, homogenize in 8 ml hypotonic KCl solution. Incubated for 40-50 minutes at room temperature. Blood was collected from fishes by caudal vein puncture. The blood was cultured at 28ºC for 120 hours, then 100µl of Colcimide as given by OSPAR (2002) was added to each flask and left to incubate for 45 minutes. The blood culture was then centrifuged at 1500 rpm for 10 minutes, supernatant was discarded. Freshly prepared hypotonic solution was added and mixed well, incubated at room temperature for 45 minutes. Hypotonic action in all the tubes containing blood and kidney tissues was stopped by adding 1 ml freshly prepared Canroy's fixative and again incubated at room temperature for 15 minutes. The cell suspension was centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded and slowly over laid with 6-8 ml of freshly prepared chilled Carnoy's fixative and centrifuged again at 1500 rpm for 10 minutes. This step was repeated 3-4 times till clear cell pellet was obtained. A drop of cell suspension was dropped onto grease free slide. The slides were then flame dried, stained with 6-8% Giemsa in phosphate buffer (pH 6.8) for 15-20 minutes. Washed with double distilled water and air-dried. The slides were screened in 100x oil immersion and the Giemsa stained metaphase spreads were imaged in a phase contrast microscope (Olympus BX51, Olympus, Japan); using Cytovision software (Applied Imaging, U.K).

Statistical Analysis: The results obtained were subjected to statistical analysis using IBM SPSS software version 21. One way ANOVA test was performed to obtain the means of chromosomal aberrations in kidney and blood tissues during sub-lethal exposure to Karanjin. The graph in Fig: 2 was made using Graph Pad Prism software version 5.

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RESULTS

The diploid chromosome number 2n of *Cyprinus carpio* is 100. **Fig: 1(a)** shows the chromosomes of blood of the control group fish, very well spread and well defined chromosomes which were very easy to count. **Fig: 1(b)** on the other hand shows the chromosomes of kidney of the control group fish, where very well spread when compared to the blood chromosomes.

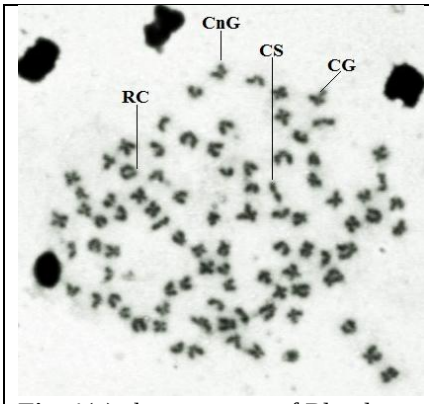

Fig: 1(a) chromosomes of Blood-control group

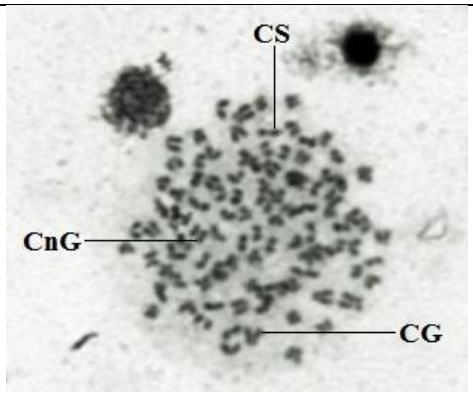

Fig: 1(b) chromosomes of kidney-control group

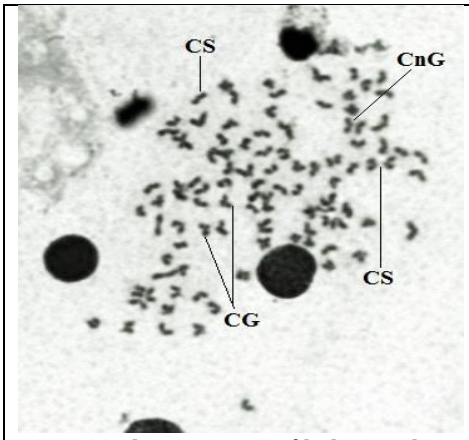

Fig: 1(c) chromosomes of kidney-24hrs exposure

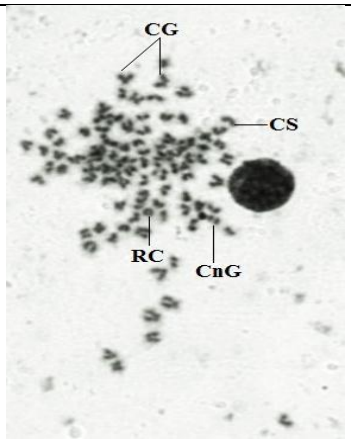

Fig: 1(d) chromosomes of kidney-7 day's exposure

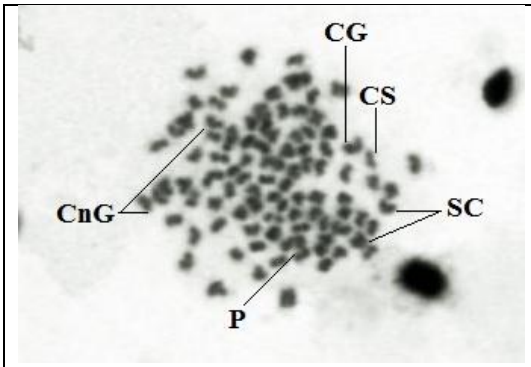

Fig: 1(e) chromosomes of kidney-14 day's exposure

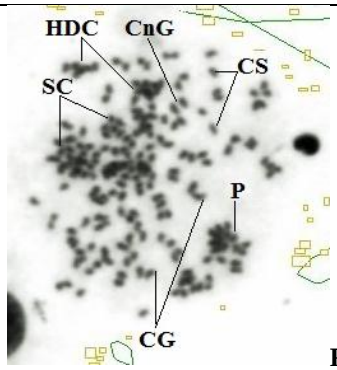

Fig: 1(f) chromosomes of kidney-21 day's exposure

CS – chromatid separation, **CG** – chromatid gap, **CnG** – centromeric gap, **RC** – ring chromosomes, **P** – pulverisation, **SC** – shorter chromosomes, **HDC** – highly distorted chromosomes.

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Table: 1 Means of chromosomal aberrations observed in blood and kidney of fish *C. carpio* during sub-lethal exposure to Karanjin.

Sr. no	Treatment group	CG	CnG	CS	RC	P	SC	HDC	Total number of aberrations	% of aberration
1	Blood control	6	3	5	1	0	0	0	15	1.5
2	Kidney Control	8	5	7	0	0	0	0	20	2
3	Kidney–24 hrs exposure	10	7	9	0	0	0	0	26	2.6
4	Kidney-7 days exposure	13	9	8	3	0	0	0	33	3.3
5	Kidney-14 days exposure	7	8	5	0	17	15	0	52	5.2
6	Kidney-21 days exposure	8	8	6	0	21	23	18	85	8.5

Number of metaphases analysed and studies = 100/treatment group.
CS – chromatid separation, **CG –** chromatid gap, **CnG –** centromeric gap, **RC –** ring chromosomes, **P –** pulverisation, **SC – s**horter chromosomes, **HDC –** highly distorted chromosomes.

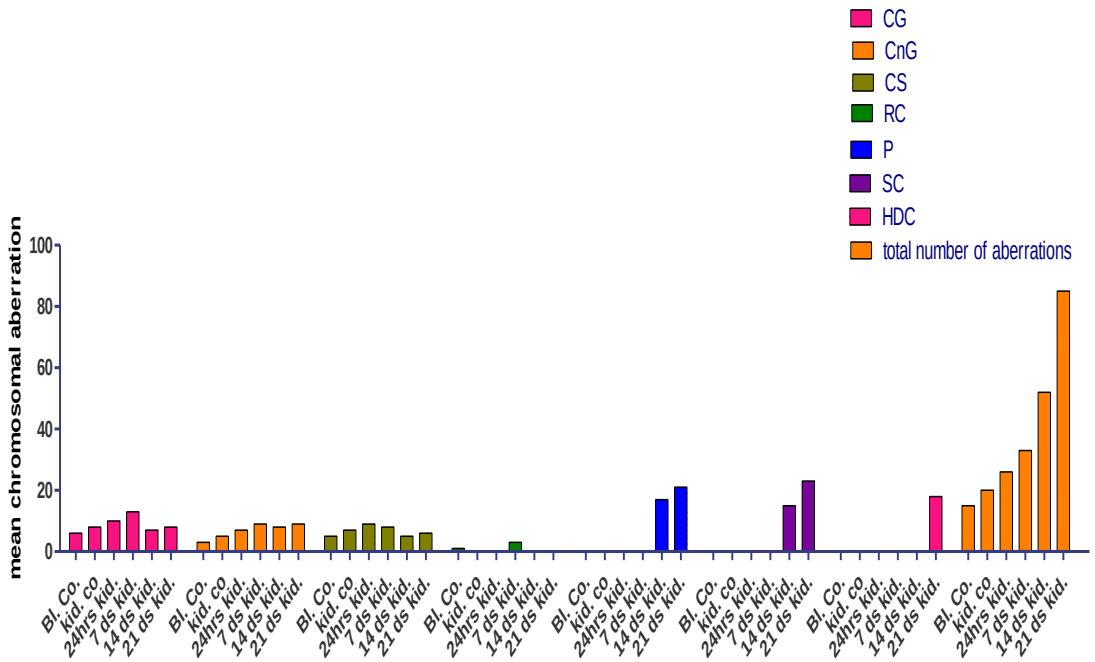

Fig: 2: Means of Chromosomal aberrations observed in Kidney and blood tissues on Exposure to Karanjin.

Blood chromosomes of control group fishes showed few aberrations which is natural, they were, centromeric gap, chromatid gap, chromatid separation and ring chromosomes. The blood showed more of blast cells (swollen cells which are very lightly stained and these are the dividing cells, which when rupture show the chromosomes at metaphase stage) and kidney showed very few blast cells. There were seen many small, darkly stained undividing cells in the kidney when compared to the blast cells. The kidney chromosomes of the control group also showed aberrations litter more than the blood, the aberrations seen in kidney control group were centromeric gap, chromatid gap and chromatid

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separation. The blood cultures were obtained for all the sub-lethal exposure periods, but none of them showed the chromosome spreads, instead there were formed many lump like structures in the test tubes, as the sub-lethal exposure period increased, the size and amount of lumps also increased, this might be due to the fact that Karanjin interferes with the cell division of blood and disrupts the cell cycle.

The kidney during the sub-lethal exposure period showed significant changes in the chromosomes. As the sub-lethal exposure period increased there were seen more of-small, darkly stained, undividing cells and very few blast cells. This indicates that during the sub-lethal exposure to karanjin, the mitotic index of kidney decreased. **Fig: 1(c)** shows chromosomal aberrations after the completion of 24hrs sub-lethal exposure period, where more of chromatid gaps, centromeric gaps and chromatid separations, as compared to the control were observed. **Fig: 1(d)** shows chromosomes of fish after 7 days exposure, still higher number of CG, CnG, CS and there were seen few ring chromosomes. **Fig: 1(e)** shows chromosomes of fish after 14 days exposure, along with CG, CnG, and CS there were seen, shorter chromosomes and also pulverisation of chromosomes. **Fig: 1(f)** shows the chromosomes of fish after 21 days of exposure there were seen along with CG, CnG, CS, SC, P, highly distorted chromosomes. **Table: 1** and **Fig: 2** shows the means of chromosomal aberrations in blood of control group fish, means of chromosomal aberrations in kidney of control and exposed group fish. As the exposure period increased the intensity of aberrations also increased. It was also observed that during the sub-lethal exposure period the chromosomes were seen in the late metaphase state, this indicates that Karanjin shows a significant effect on the cell cycle – by delaying the cell cycle time.

Fishes are used as very useful and sensitive biomarkers to assess the contamination and changes in the aquatic environments that directly and indirectly affects the health of the aquatic organisms. Mahrous and Abdou (2001) have studied that the pollution of aquatic environments by agricultural and industrial wastes have significant effects on fish species such as *Oreochromis niloticus* and *Clarias lazera*, causing chromosomal aberrations such as chromosomal breaks, chromatid deletions and centromeric attenuation in somatic cells. Mohamed *et al*. (2008) studied the cytogenetic changes in the gill cells of *Oreochromis niloticus* treated by copper sulphate and lead acetate by measuring the frequency of chromosomal aberrations. Iji and Odeogan (2014) used cytotoxicity testing as a biomarker tool in assessing and monitoring aquatic pollution in water bodies caused by effluents by measuring the frequency of chromosomal aberrations, in wild fish species *Clarias pachynema.*

The change in chromosome structure indicates a direct effect on the genetic material of an animal. By examining the chromosome structures at metaphase, after being exposed to any kind of pesticide or toxicant, the genotoxic nature of the pesticide or toxicant can be detected was suggested by Ali *et al*. (2009). The results of chromosomal aberrations observed in the present study are also in agreement with the work done by Rishi and Haobam (1990); Rishi and Grewal (1995); Saxena and Rana (2008) i.e., the chromosomal aberrations observed in the present study were also observed by these researchers in tissues of fish species exposed to different pesticides. The present results on chromosomal aberrations are also in accordance with the observations made in fish species like *Anabas* by Biswas and Manna (1989). In the present study there were seen prominent changes in the structure of chromosomes, such as decrease in length, pulverisation and over all distortion in the structure of chromosomes. The results of the present study clearly indicate that the frequency of chromosomal aberrations such as chromatid separation, chromatid gap, centromeric gap, ring chromosomes, pulverisation, **s**horter chromosomes, highly distorted chromosomes were higher in the exposed group fishes (Fig: 1c, 1d, 1e and 1f) when compared to the control group (Fig: 1,a and Fig: 1b).

The chromosomal aberrations were in less frequency during the initial period of exposure but as the duration of sub-lethal exposure increased, the frequency and degree of aberrations also increased was reported by Manna and Mukherjee (1986). Mathew and Jahageerdar (1999) have reported similar observations in *Channa punctatus* exposed to heavy metals. Our results obtained in the present study are in agreement with Yadav and Trivedi (2009) who found that the exposure of *Channa punctatus* to different types of heavy metals such as mercuric chloride, arsenic trioxide and copper sulphate pent hydrate for a week showed chromatid and chromosome breaks, chromatid and chromosome gaps, along with ring and di-centric chromosomes in the kidney cells, this indicates the genotoxic potential of these metals even in very low concentrations during sub-lethal exposure period. Bioaccumulation and bio magnifications of heavy Metals and their persistence in aquatic environment were studied by Malla and Ganesh (2009). Many investigators have

studied and proved that pollutants or toxicants of any kind in an aquatic environment could act on the aquatic life directly in the form of free oxygen radicals, which can initiate degenerative processes and cause genotoxic effects. The results in the present study show that Karanjin, though a plant secondary metabolite caused cytotoxicity (chromosomal aberrations) in common carp - *Cyprinus carpio* following sub lethal exposure. This shows that pollutants if present even in a very low concentration in environment for a long duration of time may affect the organisms at genetic level and overall health of fish may also get affected. Hence the use of all kinds of pesticides in agriculture and aquaculture fields, whether obtained as plant secondary metabolites or having a synthetic origin should be used carefully, in required amounts and under proper guidance.

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