Original Article

Induction of Micronuclei and Erythrocytic Nuclear Abnormalities in Peripheral Blood of Fish *Cyprinus carpio* on Exposure to Karanjin

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ABSTRACT

Background: We have come across the plant secondary metabolites having pesticidal properties being used in the form of pesticides. In this study, we used one of newly available and plant metabolite used as pesticide in wide agricultural fields – Karanjin, obtained from seeds of plant *Pongamia pinnata*.

Methods: The study was conducted during the month of March 2016 at the Department of Zoology, Osmania University. The fish common carp- *Cyprinus carpio* was exposed to sub-lethal concentration of karanjin i.e., 1/10th of 96 h LC50 value (0.28 ppm) for a period of 21 d. Moreover, at 24 h, 7 d, 14 d and 21 d, the peripheral blood of both control and exposed group fishes were studied for the presences of micronuclei and other nuclear abnormalities.

Results: The micronuclei were completely absent, i.e., were not seen during the sub-lethal exposure period. There were seen nuclear abnormalities such as blebbed nuclei (BN), notched nuclei (NN), differently shaped nuclei (DSN), pear-shaped nuclei (PSN), circular nuclei (CN), lobed nuclei (LN) and Karyolysed nuclei (KN). The control group showed few nuclear abnormalities.

Conclusion: During the sub-lethal exposure, as the days of exposure increased, the types of aberrations and their number also increased.

Keywords: Blood, Common Carp, Micronucleus, Nuclear Abnormality.

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INTRODUCTION

Various kinds of agricultural pesticides are widely being used for decades in order to get better protection against different type of pests. As a result of the increased use of synthetic pesticides and also the harmful effects caused by the residual effects of the synthetic pesticides, the researchers have found out a new method to eradicate the pests that is by the use of plant-derived secondary metabolites used as such or in the form of botanical pesticide. Various parts of neem tree have been used since decades as pesticidal agents, nowadays, in market we get various pesticides having been manufactured from secondary metabolites derived from seeds of neem tree. There are thousands of such plants available in nature having very good pesticidal properties, one of such plant species recently gained popularity is *Pongamia pinnata* commonly known as karanj plant, this plant has been proven to have seeds that produce secondary metabolites having natural pesticidal properties. Nowadays secondary metabolites extracted from seeds of karanj plant *P. pinnata* are being used in the manufacture of botanical pesticides with trade name derisom. It is being used in wide variety of crop fields and it is effective against different kind of insect pests. Ultimately whichever type of pesticide is used, be it synthetic or botanical, pesticides and other substances used in agricultural fields ultimately find their way into the aquatic environment through agricultural runoff and other means, which may cause some kind of ill effects of health effects to the aquatic organisms [1].

In any type of aquatic ecosystem, fish serves as the most important aquatic organism as it acquires an important position in the food chain and it is the most important source of protein for human consumption. As the fishes are inhabitants of aquatic environment, they cannot escape from the detrimental effects of the various kinds of pollutants and toxicants that reach the water body. Hence, these points make fish very important bioindicators of environmental pollution, especially of aquatic ecosystems [2]. The fish *Cyprinus carpio* used in the present study is second highly consumed species next to carps, it easily adapts to the laboratory

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environment and also feeds well on artificial fish feed.

Studies conducted in order to evaluate the changes at the level of structure of nucleus in the form of erythrocytic nuclear abnormalities [3]. Alterations in the erythrocytes by formation of micronuclei (MN) [4] are very simple, reliable, convenient and sensitive methods that have been in use for many years to assess the mutagenicity of different kinds of pollutants and toxicants persistent in the environment [5]. Nuclear abnormality was considered only when the nucleus of the erythrocytes showed change in the shape of nucleus from the normal elliptical shape [6]. Work regarding the detailed description about the nuclear abnormalities was done [7]. Micronuclei were small structures present within the cytoplasm of the erythrocytes. The characteristic features for the identification of micronuclei are – they are round or oval with a well-defined outline, stain similar to that of the main nucleus and size ranges from 1/3rd to 1/20th of the main nucleus [5]. The micronucleus consists of small chromatin fragments formed because of chromosome breaks or whole chromosomes that show unequal distribution or unequal separation during anaphase [8].

The present study was conducted to see whether the botanical derived secondary metabolite Karanjin induced the formation of micronuclei or any other kind of erythrocytic nuclear abnormalities in the peripheral blood of fish – *C. carpio*.

MATERIALS AND METHODS

The study was conducted during the month of March 2016 at the Departmental Animal House Facility, Department of Zoology, Osmania University. Juveniles of fish species *C. carpio* were bought from aquaculture pond that was having pollution-free water. The fishes were maintained at the animal house provided by the department in well-aerated tanks for a period of one month. They were fed twice daily. The 96 h LC50 value of Karanjin (Derisom) was already estimated as 2.8 ppm. 1.10th of the 96 h LC50 value i.e., 0.28 ppm is taken as the sub-lethal value. The fishes were exposed to the sub-lethal concentration for a period of 21 d. The micronucleus test was performed according to the method [5, 9]. After the completion of 24 h, 7 d, 14 d, and 21 d blood were collected through the caudal vein puncture using a syringe from both the control and exposed group fishes. A drop of blood was immediately placed on a clean slide and a thin smear was made, from each fish, 6 slides were made. The blood-smeared slides were allowed to air dry completely for 24 h, the slides were then fixed in absolute methanol for 15 min. The slides were allowed to completely dry. The slides were then stained in 8% Giemsa stain for 20 min, the slides were washed with distilled water, air dried completely. The slides were observed and analyzed fewer than 100 x oil immersion magnification, the slides was photographed and was scored for micronuclei and another type of erythrocytic nuclear abnormalities. Overall, 2000 cells were analyzed for each specimen.

Karanjin is an alkaloid, secondary metabolite isolated from the seeds of the plant *Pongamia pinnata* also known as *Derris indica.* A well-known example is azadirachtin that is a secondary metabolite derived from neem tree, in the similar way now much research has been done on *P. pinnata* and researchers found this plant as boon similar to neem tree. *P. pinnata* seeds along with producing secondary metabolites having pesticidal properties. It is also used in the manufacture of bio-diesel along with other plant species *Jatropha*. Karanjin has pesticidal properties. The source of Karanjin is derision that is a biopesticide in liquid formulation having Karanjin and Karanjin oil as active ingredients dissolved in solvents and emulsifier that are inactive.

The source of Karanjin – derisom was procured from the manufacturer, Agri life India Private Limited, IDA, bollaram, Hyderabad. Acute toxicity or 96h LC50 value of Karanjin based biopesticide derisom to the common carp – *C. carpio* was already determined by Finney's probit analysis method.

The Institutional Animal Ethical Committee guidelines have been followed and the experiment has been conducted after the approval from the Institutional Animal Ethical Committee.

The data were analyzed by one-way ANOVA using IBM SPSS software ver. 21 (Chicago, IL, USA). The results are presented as mean ± standard deviation at $P<0.05$ level of significance. The graph was made using Graph Pad Prism software version 5.

RESULTS

The fish *C. carpio* on exposure to sub-lethal concentration of Karanjin (0.28 ppm) showed negative results for the micronucleus test, i.e., none of the exposure days throughout the 21-day exposure period showed the presence of micronuclei. On the other hand, there were seen changes in the shapes of the erythrocytes in peripheral blood. The frequency of erythrocytic

nuclear abnormalities is expressed as number of abnormalities per thousand cells (%). The results are depicted in a tabular form (Table 1). The blood of the control group showed the presence of normal erythrocytes, with elliptically shaped cell packed with hemoglobin and elliptically shaped nucleus (Fig. 1).

Table 1. Frequency (‰) of MN and ENA in peripheral blood smear of fish *Cyprinus carpio* during sub-lethal exposure to Karanjin. Values expressed as mean ± Standard deviation $^{*}P<0.05$, ns-Non significant.

Type of Erythrocytic Nuclear Abnormality	Control	24 h exposure	7 d exposure	14 d exposure	21 d exposure
Micronuclei	0	0^{ns}	0^{ns}	0^{ns}	0^{ns}
Blebbed Nuclei	0.58 ± 0.07	$1.22 \pm 0.05^{*}$	$2.19 \pm 0.05^{*}$	$3.1 \pm 0.06^{*}$	$4.49 \pm 0.07^{*}$
Notched Nuclei	0.3 ± 0.05	$0.8 \pm 0.06^{*}$	$1.68 \pm 0.07^{*}$	$2.11 \pm 0.06^{*}$	$3.09 \pm 0.07^{*}$
Different Shaped Nuclei	0.12 ± 0.04	$0.29 \pm 0.05^{*}$	$0.69 \pm 0.07^{*}$	$1.07 \pm 0.05^{*}$	$0.63 \pm 0.06^{*}$
Pear Shaped Nuclei	0	$0.39 \pm 0.07^{*}$	$0.87 \pm 0.05^{*}$	$0.99 \pm 0.04^{*}$	$0.44 \pm 0.06^{*}$
Circular Nuclei	0	$0.29 \pm 0.04^{*}$	$0.62 \pm 0.06^{*}$	$0.78 \pm 0.06^{*}$	$0.82 \pm 0.07^{*}$
Lobed Nuclei	0	0^{ns}	0^{ns}	$0.49 \pm 0.07^{*}$	$0.22 \pm 0.06^{*}$
Karyolysed Nuclei	0	0^{ns}	0^{ns}	$1.50 \pm 0.08^{*}$	$5.62 \pm 0.07^{*}$
Total Number of ENA(‰)	1.01 ± 0.07	3 ± 0.26	6.07 ± 0.26	10.06 ± 0.36	15.34 ± 0.34

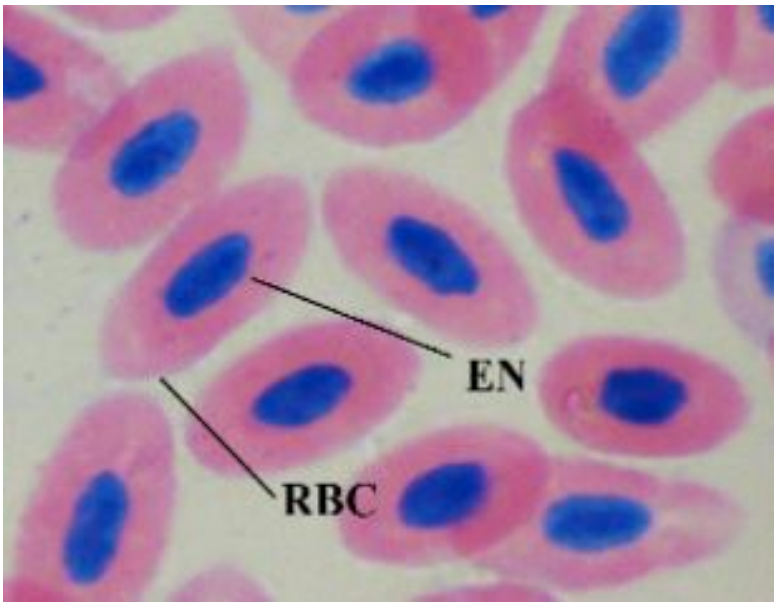

Figure 1. Normal RBC, EN-Elliptical Nucleus.

There were seen different kinds of erythrocytic nuclear abnormalities in the peripheral blood of both control group and exposed group fishes. The control group fishes showed very little type of abnormalities with less frequency. The fishes during sub-lethal exposure period showed different kinds of erythrocytic nuclear abnormalities, as the exposure period increased the type of nuclear abnormalities and their frequencies increased. Some of the erythrocytic nuclear abnormalities seen in the present study are Blebbed Nuclei (Fig. 2) in which nucleus had a small invagination in its membrane, Notched Nuclei (Fig. 3) where nucleus has deeper invagination, Differently Shaped Nuclei (Fig. 4) where the nucleus showed different shapes that were not normal, Pear-Shaped Nuclei (PSN) (Fig. 5) in which nucleus had a pear-like shape in appearance, Circular Nuclei (Fig. 6) where nucleus was circular rather than the normal elliptical shape, Lobed Nuclei (Fig. 7) in which case nucleus was lobed i.e., bilobed and Karyolysed Nuclei (Fig. 8) which showed nucleus which was seen little pale in color with vacuolated faded regions in between and it looks as if the nuclear material i.e., the chromatin material is affected, or is damaged, it gives the appearance as if the nucleus is being broken down from within. The control group fishes showed very few erythrocytic nuclear abnormalities such as Blebbed Nuclei, Notched Nuclei and Differently Shaped Nuclei (DSN).

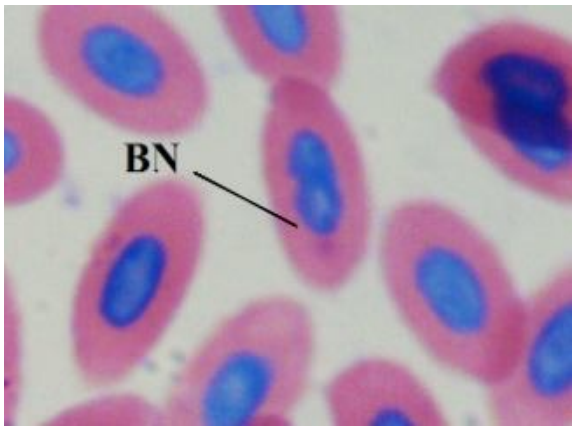

Figure 2. BN-Blebbed Nucleus.

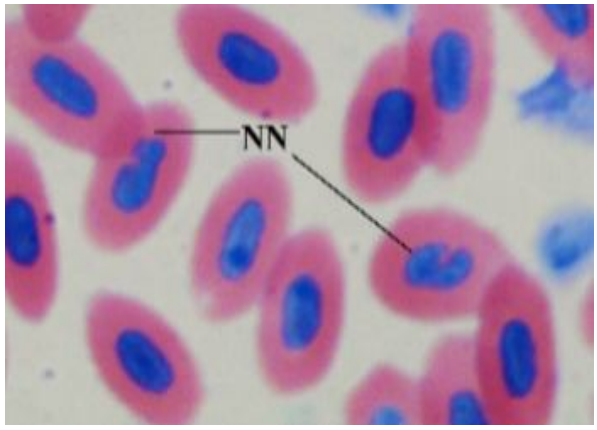

Figure 3. NN-Notched Nucleus.

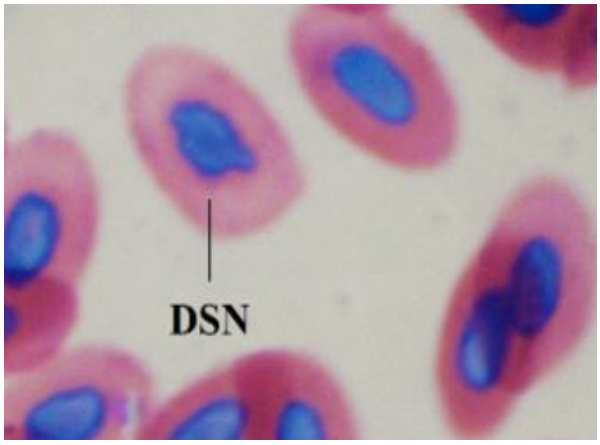

Figure 4. DSN-Differently Shaped Nucleus.

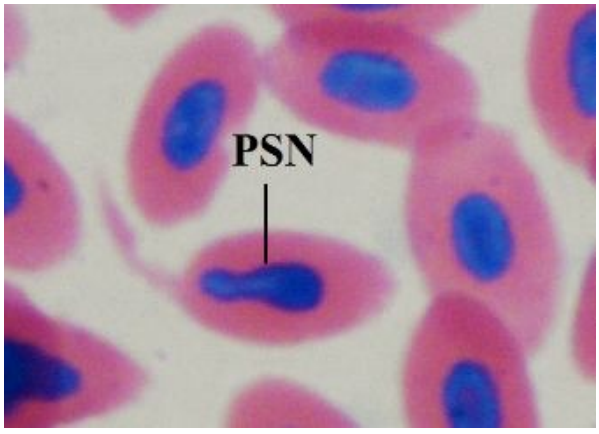

Figure 5. PSN-Pear Shaped Nucleus.

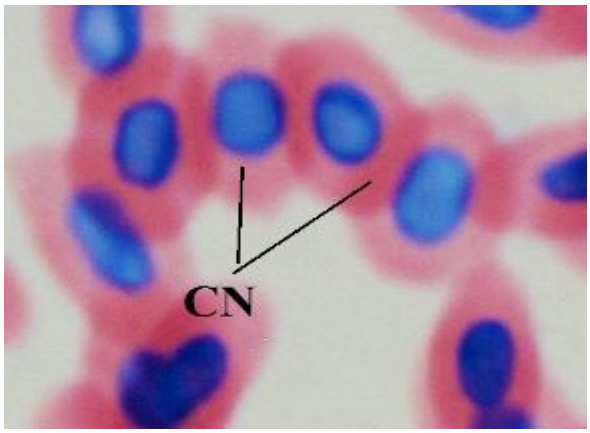

Figure 6. CN-Circular Nucleus.

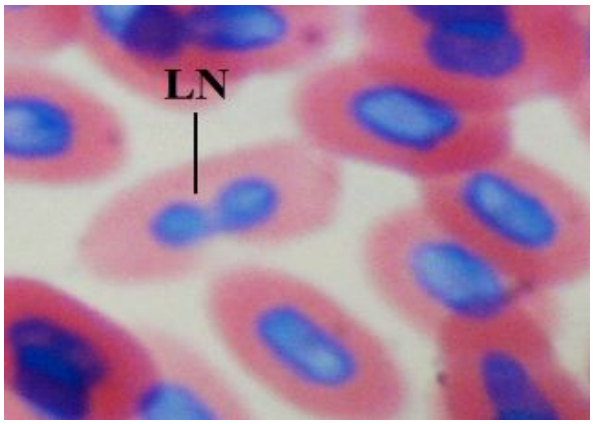

Figure 7. LB-Lobed Nucleus.

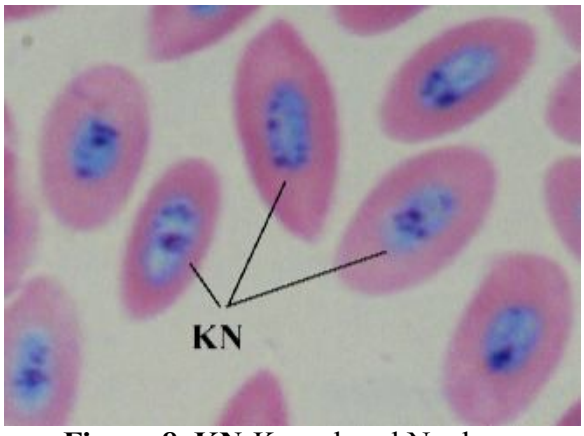

Figure 8. KN-Karyolysed Nucleus.

There were seen marked and significant (P<0.05) erythrocytic nuclear abnormalities during the sub-lethal exposure period. Compared to the control group fishes, the fishes after the completion of 24 h sub-lethal exposure showed higher frequency of Blebbed Nuclei, Notched Nuclei, DSN, along with these there were also seen other abnormalities such as, PSN and Circular Nuclei. After the completion of 7 d of sub-lethal exposure, there were seen similar erythrocytic nuclear abnormalities like that seen in the 24 h exposure period but with slightly higher frequency. The 14 d exposure period showed similar erythrocytic nuclear abnormalities like that of the 7 d exposure but with still slightly higher

frequency and along with those, there were seen other abnormalities like lobed nuclei and Karyolysed Nuclei. The 21 d exposure showed all the seven types of erythrocytic nuclear abnormalities with highest frequency of Blebbed Nuclei, Notched Nuclei, Circular Nuclei and Karyolysed Nuclei being the highest in number. The other type of nuclear abnormalities like DSN, PSN and Lobed Nuclei were seen in little lesser frequency compared to the 14 d exposure period. As the sub-lethal exposure period increased there was seen an increase in the frequency (%) of a total number of erythrocytic nuclear abnormalities (Fig. 9).

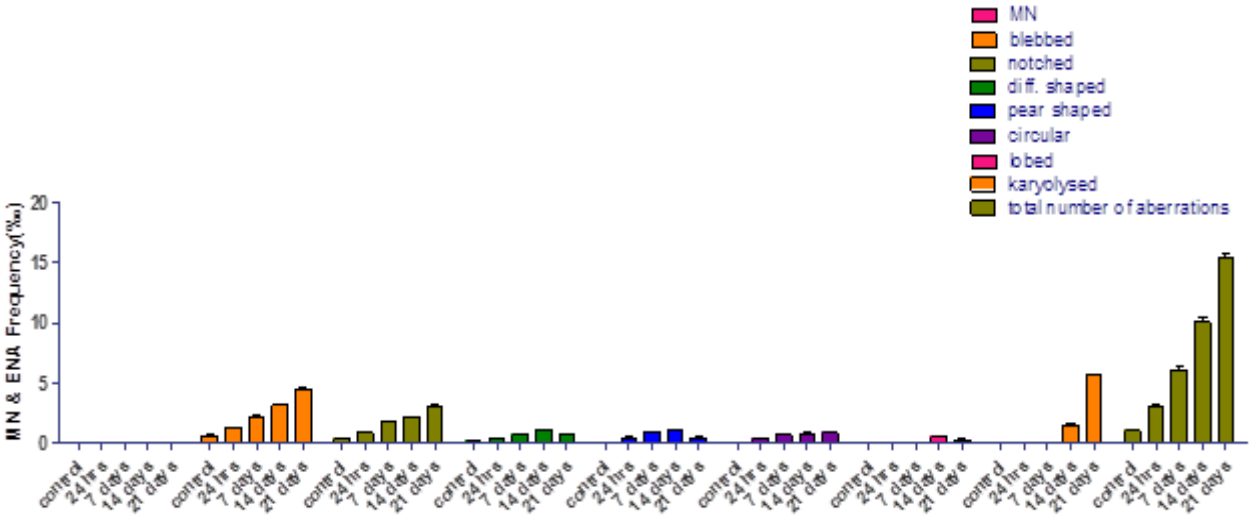

Figure 9. MNT and other nuclear abnormalities in peripheral blood of fish - *C. carpio* during sub-lethal exposure to Karanjin.

DISCUSSION

The present study showed that the fish *C. carpio* showed zero frequency of micronuclei in the control group as well as in the groups exposed to sub-lethal concentration of Karanjin. Similar kinds of results were also seen by some other researchers [10]. None of the sub-lethal exposure days – 24 h, 7 d, 14 d and 21 d showed the formation of micronuclei. Karanjin showed its effect on the structure and shapes of the nuclei of red blood cells but it did not cause much harm or damage to the genetic make-up of the cells of the organism that could otherwise result in the formation of micronuclei. Hence, Karanjin showed erythrocytic nuclear abnormalities in the peripheral blood of *C. carpio* and the micronuclei were absent in both control and exposed group fishes.

The micronucleus test and the erythrocytic nuclear abnormalities are considered as powerful tools for monitoring the environment for the presence of genotoxic agents. The frequency of micronuclei has been proved very reliable test for studying genotoxicity in vivo and in vitro hence making it possible to compare the results obtained in the laboratory with that in the natural ecosystem. In the present study, there were no micronuclei seen either in the control or in the exposed group fishes, Karanjin causes only a few nuclear abnormalities in the nucleus of erythrocytes but did not form micronuclei. The actual and exact mechanism for the formation of erythrocytic nuclear abnormalities is not fully understood [8].

The results of studies conducted with erythrocytic nuclear abnormalities provide us very strong evidence that they are very important and effective genotoxic markers especially considered while studying genotoxicity related studies mainly in freshwater fishes, whether studied in wild or in laboratory, under controlled environmental conditions [9, 11]. It is always a necessity and an important aspect to test and study the aspect of genotoxicity – micronuclei or erythrocytic nuclear abnormality in the control group fishes because it gives us a clear result and conclusion that whatever changes related to genotoxicity are seen in the exposed group fishes is due to the test compound or the toxicant.

There is always a variation between the data obtained from the work done in the laboratory conditions and that obtained directly from the studies conducted in the natural ecosystems, because the physical, chemical and biological factors of any aquatic ecosystem are always integrated with each other. Hence always the results obtained to assess any kind of genotoxicity it the

laboratory conditions will show a slight variation because no human can replicate the similar environment in the laboratory like that of the nature. Furthermore, standard laboratory conditions are always slightly having variations when compared to the natural environmental conditions [12]. Much research has been done on the formation of micronuclei in the erythrocytes of fishes collected from polluted water bodies, used as an important marker for genotoxicity studies [5, 13] and to assess the genotoxicity of various components [4, 7,14, 15]. Some authors have worked on different fish species to evaluate the genotoxicity by using micronucleus test [13]. The present study has very clear results showing absence of micronuclei in both the control and exposed group fishes.

However, there is a very clear relation and difference seen when compared the erythrocytic nuclear abnormalities in the control and exposed group fishes. There is a very clear relation between the nuclear abnormality of erythrocytes and the exposure period of fishes to Karanjin. The concentration of Karanjin during the sub-lethal exposure was 0.28 ppm, but as the exposure period increases the frequency of nuclear abnormalities in the exposed group fishes also increased. There are many kinds of pollutants that reach the aquatic habitats through run-off, some of the most important pollutants are the one being used in the agriculture and aquaculture fields example various kinds of pesticides, oil, and grease, nutrients [16], total hydrocarbons, PHAs and heavy metals [17]. Every day there are large quantities of pollutants and toxic substances considered as wastes present in the industries and urban effluents and from the agricultural fields are reaching the ponds, rivers and the sea. Some of the pollutants and toxicants present in these effluents might contain organic, inorganic and metallic substances that may be potential genotoxic substances.

Regarding the increase in the nuclear abnormality of erythrocytes during the sub-lethal exposure as the days of exposure increased are in agreement with many of the other researchers demonstrated the detection of nuclear abnormalities in the erythrocytes of various fish species. During the sub-lethal exposure period as the days of exposure increased there was increase in the frequency of Karyolysed nuclei seen in 14 d exposure and was highest on 21st day of exposure, other nuclear abnormalities that showed increase in their frequency during sub-lethal exposure were blebbed nuclei and notched nuclei, some of the workers have studied the genotoxic response in various fish species on exposure to crude oil and also to different PAHs [14, 18-20]. Similarly, also nuclear abnormalities have been studied by researchers when fish species were exposed to petroleum and other distillate products [21] and to other toxicant compounds [9, 22, 23]. The red blood cells of the peripheral blood were the main target cells studied by majority of the researchers. Not only in the erythrocytes obtained from the peripheral blood, but nuclear abnormalities were also observed in the erythrocytes obtained from kidney of different freshwater fish species on exposure to different kinds of clastogenic and aneugenic compounds [9, 23].

CONCLUSION

Karanjin showed zero results for micronuclei but some structural abnormalities were seen at the level of nuclei of red blood cells in the fish common carp – *C. carpio* during sublethal exposure to Karanjin (0.28 ppm). Pollutants even in a very low concentration if present for a long duration may affect at the level of nucleus. Hence, the use of any kind of substances such as natural plant metabolites or other natural and synthetic chemicals in agriculture and aquaculture field should be carefully monitored and pesticides and other substances should be used under proper guidance.

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