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Evaluation of genotoxicity by comet assay (single-cell gel electrophoresis) in tissues of the fish *Cyprinus carpio* during sub-lethal exposure to Karanjin

Shoeiba Tasneem* and Rafath Yasmeen

Abstract

Background: Adverse effects caused by the synthetic pesticides has forced scientists to develop an alternative against the synthetic pesticides. Therefore, it has come a boon to mankind in the form of neem tree having secondary metabolites showing pesticidal properties which is in use since many years. Like neem tree, there has been research done on karanja plant, and its secondary metabolite karanjin has been found to possess good pesticidal properties. Comet assay or alkaline single-cell gel electrophoresis (SCG) was performed in fish *Cyprinus carpio* after exposure to sub-lethal concentration (0.28 ppm) of karanjin. The assay was carried out in the gill, liver, kidney, and blood. The total DNA content in each tissue was also estimated using Nano Drop.

Results: The present study clearly shows that though karanjin is a derived secondary metabolite from plant, it is capable of producing genotoxicity in common carp. As the days of sub-lethal exposure increased, all the four tissues showed decrease in total DNA content which was directly proportional to the formation of DNA comet tails.

Conclusion: The use of any kind of plant metabolites in the agriculture and aquaculture farms should be monitored well.

Keywords: Gill, Liver, Kidney, Blood, SCGE, Common carp

Background

The human populations around the globe have faced many problems and also harmful health effects due to the residual effects of synthetic pesticides. Since the past few decades, many scientists have done a lot of research in searching and formulating an alternative for the synthetic pesticides. A big problem of humans has been solved by the discovery of plants possessing natural insecticidal components (Lopez, Jordan, & Pascual-Villalobos, 2008; Pavela, 2009; Kim et al., 2010). Plants are the inexhaustible sources of structurally diverse and biologically active substances having medicinal and pesticidal properties (Istvan, 2000). There are many plant species available throughout the world. Different plant parts such as leaves, seeds, bark, and stem are being used either in the crude form or as active ingredients which are being isolated and being used as pesticides (Karunamoorthi, 2012).

Neem tree is known since ancient times to have medicinal and pesticidal properties. Every part of neem tree contains various classes of active ingredients that have insecticidal properties, piscicidal properties, and molluscicidal properties. Various parts of neem tree have been used in many countries as insecticides and insect repellents apart from many other medicinal uses (WHO, 2003). The active ingredient which has been isolated from the seeds of neem is azadirachtin, and it has been used since many years in the manufacture of many neem-based pesticides with different concentrations and different names (Anis Joseph, Premila, Nisha, Rajendran, & Sarika Mohan, 2010; Vethanayagam & Rajendran, 2010). We have an assumption that as these active

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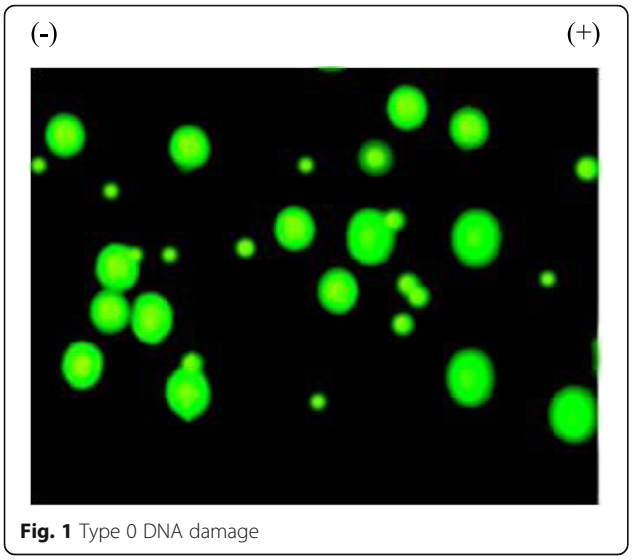

Fig. 1 Type 0 DNA damage

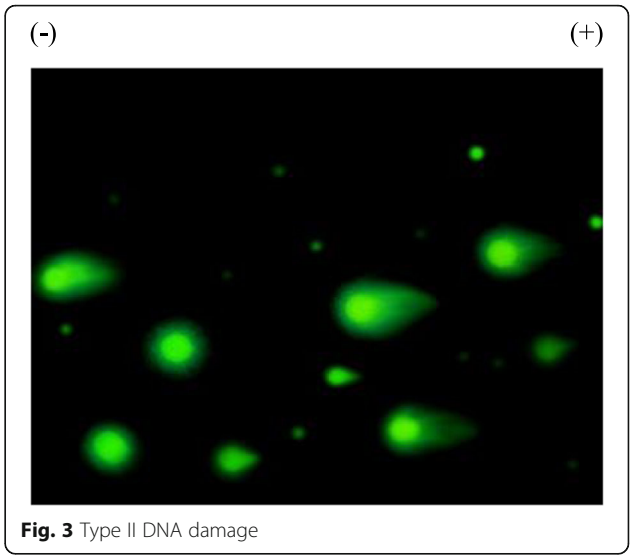

Fig. 3 Type II DNA damage

metabolites are synthesized and isolated from plants, they are very safe and not having any impact on other non-target organisms. Recently, it was found that a neem-based pesticide, with brand name Achook, was toxic to zebrafish *Danio rerio* (Ansari & Sharma, 2009).

Not only neem tree but there are a thousand many more trees having active components possessing pesticidal properties. One more plant species that has gained attention and interest to scientists is the *Pongamia pinnata*, also known as *Derris indica*, commonly known as karanj plant. One of the most important active ingredients isolated from the seeds of this plant is a flavonoid called karanjin. This active ingredient is being used in the manufacturing of biopesticides and bioinsecticides in different names, one of them being Derisom (Majumdar, 2002; Majumdar, Pandya, Arora, & Dhara, 2004; Tamrakar, Yadav, Tiwari, Maurya, & Srivastava, 2008; Al Muqarrabun, Ahmat, Ruzaina, Ismail, & Sahidin, 2013). The seeds of *Pongamia pinnata* have an additional important benefit along with its possessing pesticidal properties, and the seeds are also being used for the synthesis of biodiesel along with the famous plant *Jatropha*. Hence, *Pongamia pinnata* can be considered as a boon to mankind next to neem tree.

Comet assay or alkaline single-cell gel electrophoresis (SCGE) is a very simple, fast, and sensitive technique for assessing genotoxicity by quantifying the amount of DNA damage caused in individual cells. It is used as an important tool for environmental monitoring and assessing health of aquatic animals by detecting DNA damage in fish, clams,

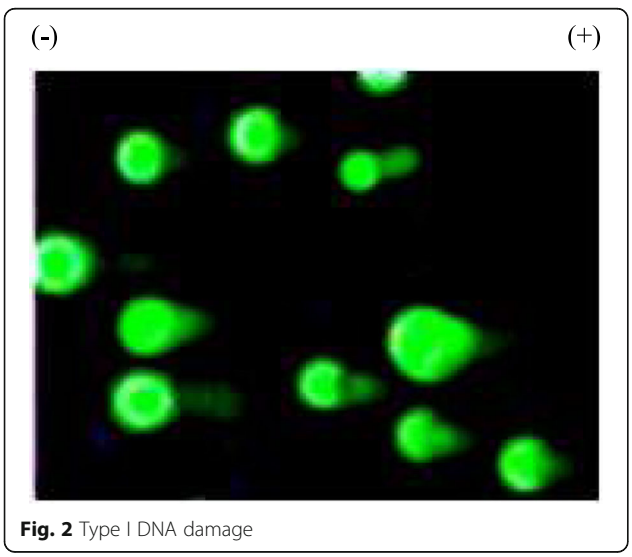

Fig. 2 Type I DNA damage

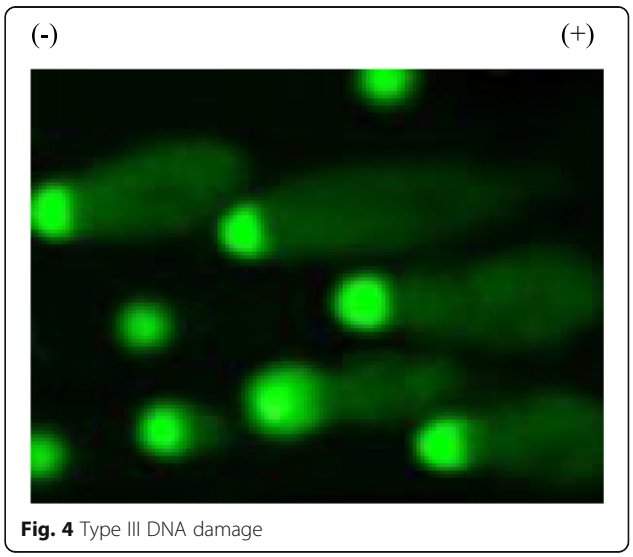

Fig. 4 Type III DNA damage

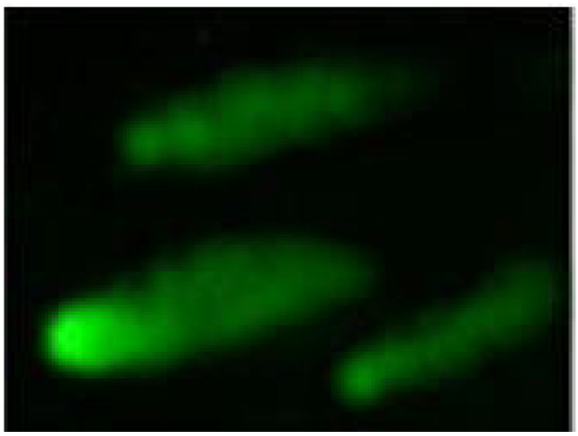

Fig. 5 Type IV DNA damage

shellfish, and mussels (Andrade, Freitas, & Silva, 2004; Belpaeme, Delbeke, Zhu, & Kirsch-Volders, 2004). Comet assay is used to detect genetic damage in the form of DNA strand break, which is an additional sensitive indicator. This assay has been applied in aquatic environments to assess and monitor the health and genetic condition of both vertebrate and invertebrate organisms (Kleinjans & Van Schooten, 2002). In vivo data on fish were obtained with redbreast sunfish (*Lepomis auritus*), hard head cat fish (*Anus felis*), bullhead (*Ameirurus nebulosus*), and carp (*C. carpio*) to see if there was any kind of genetic damage in tissues of fish in aquatic environment due to pollutants (Mitchelmore & Chipman, 1998). Comet assay studies have also been performed in *Cyprinus carpio* exposer in vitro to heavy metal nickel and lead (Mitkovska, Dimitrov, & Chassovnikarova, 2017). DNA damage studies have been performed in different fish species collected from upper and middle stretches of Adige river basin (Kracun-Kolarevic et al., 2016).

The fish used in the present study is the common carp—*Cyprinus carpio*. It is the second largest fish consumed next to Indian major carps. The fish has high growth rate, has easy adaptability to lab conditions, and easily feeds on artificial feed; hence, it has been selected for the research purpose. Till date, no other researcher has worked on the toxicity and genotoxicity effects of karanjin on *C. carpio* or any other fish species. Hence, the aim of the study was to evaluate the DNA damage in tissues of fish, *C. carpio*, on exposure to karanjin.

Table 2 Nucleoids in various damage degrees in the liver of *C. carpio* on exposure to karanjin

Exposure	Type 0	Type I	Type II	Type III	Type IV
Control	87 ± 1.41	8.33 ± 1.5	4.3 ± 1.03	0	0
24 h	80.33 ± 2.58	8.5 ± 1.04	11.16 ± 1.16	0	0
7 days	74 ± 2.6	4.8 ± 1.47	10 ± 2.09	8.16 ± 1.16	3.16 ± 1.16
14 days	63 ± 2.6	4.5 ± 1.04	6.5 ± 1.04	12.66 ± 1.63	13.5 ± 1.04
21 days	52.66 ± 2.80	3.16 ± 1.16	8.66 ± 1.75	16 ± 2.60	19.66 ± 2.16

One hundred cells are evaluated. Values are expressed as mean ± standard deviation

Methods

Source of karanjin

Karanjin is a furano-flavonoid isolated from the seeds of *Pongamia pinnata* also known as *Derris indica.* Karanjin has been proven to have pesticidal properties. The source of karanjin is "Derisom" which is a biopesticide in liquid formulation having karanjin and karanjin oil as active ingredients dissolved in solvents (butanol and butyl acetate) and emulsifier which are inactive. The source of karanjin, Derisom, was procured from the manufacturer Agri Life India Private Limited, IDA, Bollaram, Hyderabad. The acute toxicity or 96-h lethal concentration (LC)50 value of karanjin-based biopesticide Derisom to the common carp *Cyprinus carpio* was already determined by Finney's probit analysis method.

Experimental animal

Juveniles of *Cyprinus carpio* ranging in length 14 ± 0.83 cm and weighing 38.53 ± 1.79 g were collected from Kaikaluru village of Andhra Pradesh State and transported to the laboratory in a well-aerated condition. The fishes were acclimatized in well-aerated tanks for a period of 1 month. They were fed twice daily with commercially available fish feed pellets, and the water was renewed 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 concentration taken

Table 1 Nucleoids in various damage degrees in the gill of *C. carpio* on exposure to karanjin

Exposure	Type 0	Type I	Type II	Type III	Type IV
Control	86.5 ± 3.27	9.66 ± 1.03	4 ± 0.89	0	0
24 h	74.16 ± 3.06	18.66 ± 3.38	7.33 ± 1.86	0	0
7 days	66.33 ± 2.16	8.16 ± 1.47	12.5 ± 1.87	9.5 ± 1.87	3.66 ± 2.16
14 days	54.33 ± 2.80	6.5 ± 1.04	10.66 ± 1.21	17 ± 2.36	11.5 ± 2.42
21 days	50.16 ± 2.40	3.83 ± 1.47	7.83 ± 2.13	20.83 ± 1.47	17.5 ± 1.87

One hundred cells are evaluated. Values are expressed as mean ± standard deviation

Table 3 Nucleoids in various damage degrees in the kidney of *C. carpio* on exposure to karanjin

Exposure	Type 0	Type I	Type II	Type III	Type IV
Control	88.16 ± 1.47	7.33 ± 1.96	5.16 ± 1.72	0	0
24 h	77.5 ± 2.42	13.5 ± 1.87	9.33 ± 1.50	0	0
7 days	67.83 ± 2.13	8.16 ± 1.47	6.16 ± 1.16	15.16 ± 1.47	2.83 ± 0.75
14 days	60.16 ± 1.16	9.5 ± 1.04	7.66 ± 1.63	14.16 ± 1.16	8.83 ± 1.16
21 days	53.5 ± 2.16	7.83 ± 1.47	11.16 ± 1.16	13.16 ± 1.16	14.66 ± 1.75

One hundred cells are evaluated. Values are expressed as mean ± standard deviation

Table 4 Nucleoids in various damage degrees in the blood of *C. carpio* on exposure to karanjin

Exposure	Type 0	Type I	Type II	Type III	Type IV
Control	89.5 ± 3.08	6.83 ± 1.47	3.83 ± 1.47	0	0
24 h	80.83 ± 1.47	7.66 ± 1.36	11.5 ± 1.87	0	0
7 days	69.83 ± 2.31	6.5 ± 1.04	8.66 ± 1.96	9.66 ± 1.36	5.5 ± 1.87
14 days	60.5 ± 3.08	5.83 ± 1.47	10.33 ± 1.86	13.33 ± 2.25	10.16 ± 1.47
21 days	51 ± 2.09	4.16 ± 1.16	9.5 ± 1.04	16.33 ± 2.25	19.16 ± 2.13

One hundred cells are evaluated. Values are expressed as mean ± standard deviation

as the sub-lethal value. The fish were exposed to the sub-lethal concentration for a period of 21 days. After the completion of 24 h, 7 days, 14 days, and 21 days, the blood was drawn through caudal vein puncture from both control and exposed group fishes and the blood was transferred into EDTA coated tubes, an equal amount of chilled phosphate buffer was added, and centrifuged at 5000 rpm for 15 min, and the supernatant was discarded and the pellet was stored in a − 20 °C refrigerator till further analysis was done. The organs - gill, liver and kidney were dissected from the fishes of both control and exposed groups, washed in saline, and transferred to tubes containing phosphate buffer.

Protocol for comet assay

The alkaline comet assay was performed according to Tice et al. (2000), as modified by Hartman et al. (2004) and Lemos, Dias, Silva-Souza, & Mantovani (2005). Cells are combined at 1×10^5/ml with molten low-melting agarose (LM Agarose) (at 37 °C) at a ratio of 1:10 (*v*/*v*), and immediately, 50 μl of it is pipetted out onto a comet slide which was placed on a flat surface at 4 °C in the dark for 10 min. The slides (coated with LM Agarose and cell suspension) were immersed in a lysis buffer solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Trizma base) at 4 °C for 30–60 min. The comet slides were immersed in freshly prepared alkaline unwinding solution (300 mM NaOH/1 mM EDTA), pH > 13 for 20 min at room temperature. The slides were placed in the electrophoresis slide tray and covered with a slide tray overlay. The power supply was set at 21 V, and the voltage was applied for 30 min. The samples were dried at 37 °C for 10–15 min. The samples were stored at room temperature, with desiccant (commonly used desiccant is silica gel) prior to scoring. One hundred microliters of diluted SYBER Green dye was placed onto each circle of dried agarose and stained for 30 min at room temperature in the dark. They were finally allowed to dry completely at 37 °C, viewed, observed thoroughly by a fluorescence microscope (Carl Zeiss, Germany), and photographed.

Extraction of DNA from the gill, liver, kidney, and blood of *C. carpio*

Fresh tissues from individual fish were taken and DNA was extracted using a DNeasy Qiagen kit, following the tissue and blood extraction protocol provided along with the kit. Twenty-five milligrams of tissue of the gill, liver, and kidney and 10 μl of the blood were collected. One hundred eighty microliters of buffer ATL was added to each of the tube of the gill, liver, and kidney. To each tube, 20 μl of proteinase K was added in order to obtain the lysed tissue. Two hundred microliters of buffer AL was added to all tubes and mixed thoroughly by vortexing. Later, 200 μl of ethanol (96–100%) was added to the tubes and mixed again thoroughly by vortexing. The tubes were centrifuged at 8000 rpm for 1 min. Five hundred microliters of buffer AW1 was added and centrifuged for 1 min at 8000 rpm. Five hundred microliters of buffer AW2 was added and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. Two hundred microliters of buffer AE was pipetted out directly onto the DNeasy membrane, incubated at room temperature for 1 min, and centrifuged for 1 min at 8000 rpm for the DNA to elute.

Electrophoresis of the extracted DNA

The purity of the extracted DNA was estimated using agarose gel electrophoresis (Salem et al., 2014). The DNA obtained was subjected to electrophoresis at 100 V on 1.2% agarose gel with Tris-borate-EDTA buffer

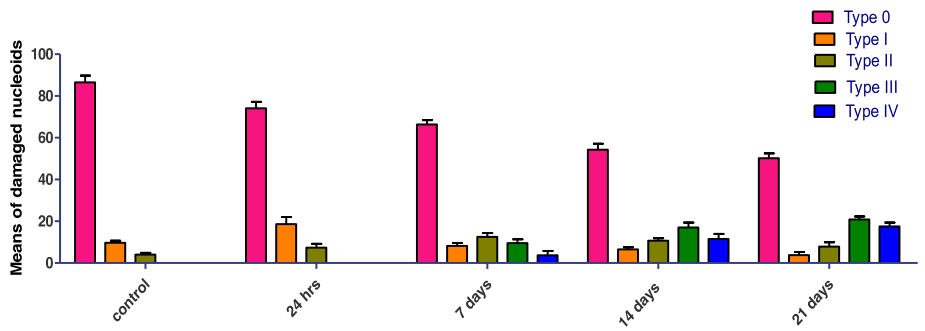

Fig. 6 Graph representing nucleoids in various degrees of damage (mean ± S.D.) in the gill of *C. carpio* during sub-lethal exposure to karanjin

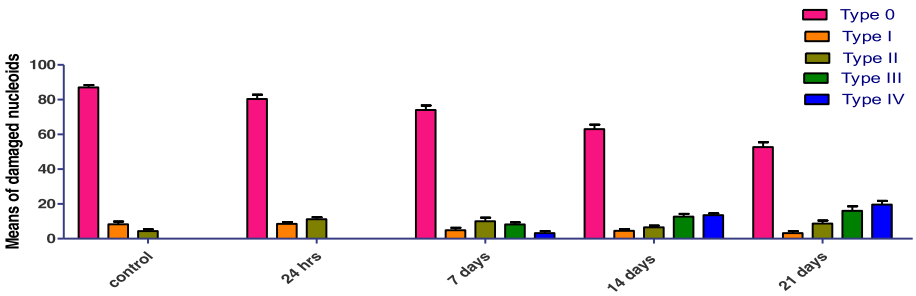

Fig. 7 Graph representing nucleoids in various degrees of damage (mean ± S.D.) in the liver of *C. carpio* during sub-lethal exposure to karanjin

(Sambrook, Fritsch, & Maniatis, 1989). The gel was placed into the tank. EtBr was added to the 900 ml of 0.5× TBE (45 μl of a 10 mg/ml stock solution). The buffer was poured into the tank covering the gel. One microliter of loading dye was added per 5-μl sample. Samples were then loaded into the wells. The tank was connected to power supply—voltage at ~ 100 V. The power is supplied for about an hour. The gel was imaged using gel doc instrument to visualize the DNA bands under UV light. The gel was carried in the casting tray and transferred onto the trans-illuminator. The UV light was turned on to 100%. The DNA bands were visible and the images were captured by the camera provided along with the gel doc instrument, and the image was saved for further analysis.

Quantification of extracted DNA using Nano Drop

The concentration of the extracted DNA in the tissue samples of both control and exposed groups was estimated using Nano Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) (Salem et al., 2014). Nano Drop is a very efficient and easy to use instrument, which requires very less amount of sample to be measured, i.e., it requires only 2 μl of DNA sample. Nano Drop uses a wavelength of 260/280 nm to measure the amount of DNA in the given sample. The values were expressed as nanograms per microliter of sample.

Statistical analysis

All the results obtained were subjected to statistical analysis using IBM SPSS software version 21 using one-way ANOVA along with post hoc LSD test. All the results are presented as mean ± standard deviation at $P < 0.05$ level of significance. The graph was made using GraphPad Prism software version 5.

Results

For each exposed and control group, a total of hundred comets were scored visually (50/slide and 2 slides/fish). The cells were categorized into five classes based on the presence of tail and the intensity of tail length. The cells having no DNA damage showed the presence of intact nuclei, and these were as follows: the first class cells of were the type 0 (no DNA damage) (Fig. 1), the second class of cells were the type I (mild DNA damage) (Fig. 2), the third class of cells were the type II (moderate DNA damage) (Fig. 3), the fourth class of cells were the type III (high DNA damage) (Fig. 4), and the fifth class of

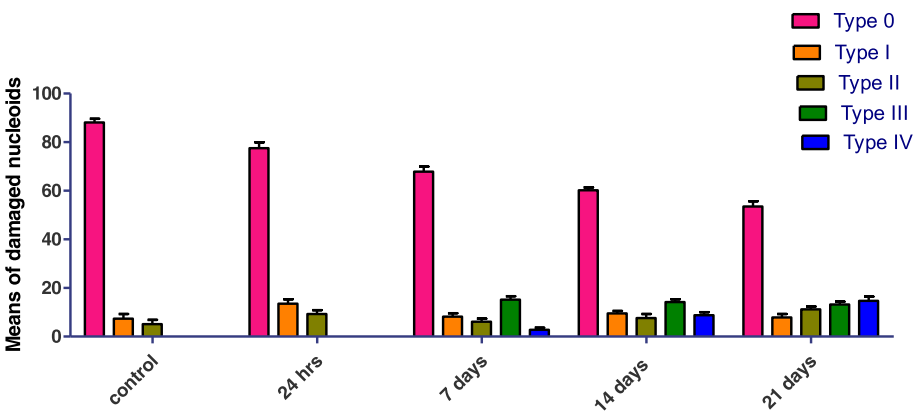

Fig. 8 Graph representing nucleoids in various degrees of damage (mean ± S.D.) in the kidney of *C. carpio* during sub-lethal exposure to karanjin

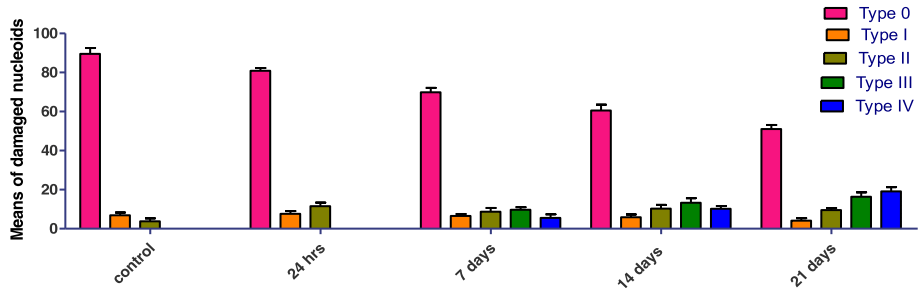

Fig. 9 Graph representing nucleoids in various degrees of damage (mean ± S.D.) in the blood of *C. carpio* during sub-lethal exposure to karanjin

cells were the type IV (extensive DNA damage) (Fig. 5). The classes of DNA damage type 0 to type IV were given by Anderson, Yu, Phillips, and Schezer (1994) (Tables 1, 2, 3, and 4). This method of comet scoring gives us a quantifiable resolution which is useful for many purposes (Liao, McNutt, & Zhu, 2009). The amount of DNA migrated as comet tail of various lengths was known as DNA damage (Grover et al., 2003). The DNA damage was evaluated as percent of damage cells (taking into account only the cells of type II, type III, and type IV) as given by Palus, Dziubaltowska, and Rydzynski (1999) and genetic damage index (GDI) as described by Collins (2004) and Pitarque, Creus, Marcos, Hughes, and Anderson (1999). The genetic damage index was calculated by using the formula:

$$\mathrm{GDI} = \frac{1.\mathrm{Type\,1} + 2.\mathrm{Type\,II} + 3.\mathrm{Type\,III} + 4.\mathrm{Type\,IV}}{\mathrm{Type\,0} + \mathrm{I} + \mathrm{II} + \mathrm{III} + \mathrm{IV}}$$

The tissues gill, liver, kidney, and blood showed the presence of maximum number of only type 0 and type I nucleoids in the control group (Figs. 6, 7, 8, and 9). The tissues of the exposed group showed the presence of type II, type III, and type IV nucleoids along with the presence of type 0 and type 1 nucleoids. The cells having type 0 and type I nucleoids are considered as undamaged cells, while the cells having type II, type III, and type IV nucleoids are considered as damaged.

In all the tissues, as the exposure period increased, the number of cells showing type II, type III, and type IV nucleoids increased. The gill and liver showed maximum damage in type IV damaged cells where complete DNA was in tail, i.e., the length of DNA comet tail was very long where the head of the nucleus was very small. The kidney and blood showed the type IV DNA damage where the DNA comet tail length was long, but here, the head of the nucleus was distinctly visible.

Percentage (%) of DNA damage in fish increased significantly in all the tissues as the days of exposure increased (Table 5). % of DNA damage was calculated by taking into account the number of nucleoids in type II + type III + type IV. Least DNA damage was seen in the tissues of control group (Fig. 10). All the tissues showed maximum DNA damage on the 21st day. % of DNA damage in tissues after 21 days of exposure was G > B > L > K.

The genetic damage index (GDI) was found to be least in all the tissues in the control group (Fig. 11). The GDI was highest in all tissues on the 21st day of exposure, i.e., as the days of exposure increased, GDI increased significantly (Table 6). The GDI was highest on the 21st day in G > B > L > K.

Concentration of DNA

The total DNA content in 1 μl of sample was calculated for all the tissues in control group, 24 h exposure, 7 days exposure, 14 days exposure, and 21 days exposure using Nano Drop ND-100 at 260/280 nm (Fig. 12 and Table 7).

Table 5 % of DNA damage in tissues of *C. carpio* on exposure to karanjin

Tissue/exposure	Control	24 h	7 days	14 days	21 days
Gill	4 ± 0.89	7.33 ± 1.86*	25 ± 5.43*	39.16 ± 4.87*	46.16 ± 4.35*
Liver	4.33 ± 1.03	11.16 ± 1.16*	21.33 ± 2.87*	32.66 ± 2.5*	44.33 ± 3.98*
Kidney	5.16 ± 1.72	9.33 ± 1.5*	24.16 ± 2.31*	30.66 ± 2.5*	39 ± 3.34*
Blood	3.83 ± 1.47	11.5 ± 1.87*	23.83 ± 4.87*	33.83 ± 3.76*	45 ± 4.42*

Values are expressed as mean ± standard deviation
*$P < 0.05$

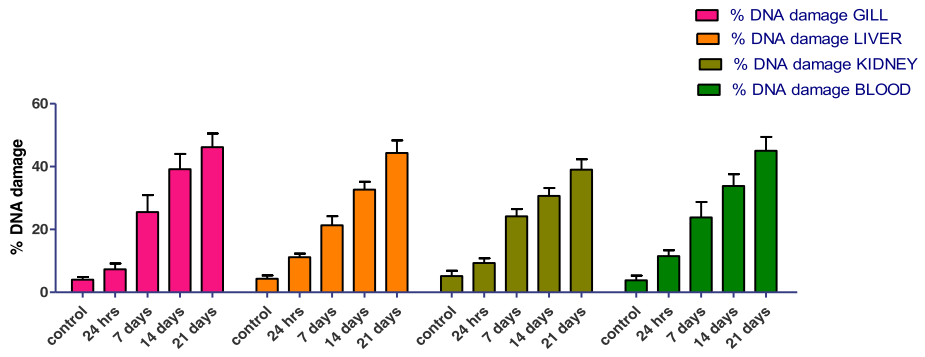

Fig. 10 % of DNA damage in tissues of *C. carpio* during sub-lethal exposure to karanjin

The amount of DNA is thus represented as nanograms per microliter of sample. The amount of DNA was found to be highest in the liver followed by the kidney, the blood, and least found in the gills, i.e., liver > kidney > blood > gill. All the tissues of control group fishes showed higher amount of DNA while the tissues of exposed group fishes showed significant decrease ($P < 0.05$) in the amount of DNA as the exposure period increased, throughout the completion of 21 days of sub-lethal exposure to karanjin (Table 5 and Fig. 9). These results are also in agreement with the gel doc images of gels having the DNA samples of the gill, liver, kidney, and blood of control group and exposure groups (Figs. 13, 14, 15, and 16). The reason for the decrease in the DNA content in all the tissues during sub-lethal exposure to karanjin may be due to the decrease in protein synthesis, defective nucleic acid metabolism, degradation of cells, inhibition of enzymes useful in replication of DNA, loss of cell structure, proliferation of new cells, formation of new tissue, and tissue degradation. Whenever there is DNA damage, the process of DNA repair mechanism also under process in a cell. The presence of karanjin or any other pesticide slows down the DNA repair mechanism.

Discussion

In the present study, observable reduction of optical density was induced by karanjin when compared with control. Fish are one of the most important aquatic organisms in any aquatic ecosystem as they play many roles in the trophic web; they respond directly to very low concentrations of toxic substances which are present in the water and also respond indirectly by feeding on contaminated aquatic organisms (Cavas & Ergene-Gözükara, 2005; Sasaki et al., 1997). Hence, fish can be used as biomarkers for early detection of contamination of an aquatic ecosystem. Pesticides form the important group of compounds concerning pollution of water bodies. Fish are best indicators of pesticide pollution in an aquatic environment due to the fact that their metabolic state changes frequently in response to pesticides (Begum, 2004; Cattaneo et al., 2011). Various species of

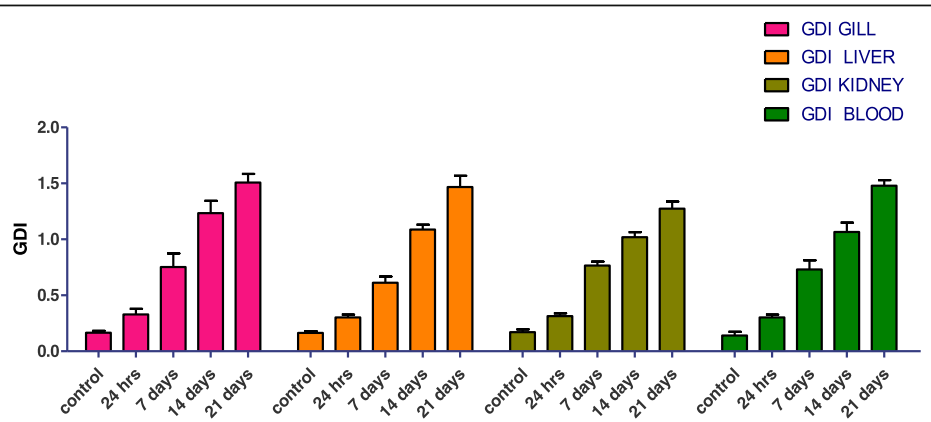

Fig. 11 Genetic damage index (GDI) in the tissues of *C. carpio* during sub-lethal exposure to karanjin

Table 6 Genetic damage index (GDI) in tissues of *C. carpio* on exposure to karanjin

Tissue/exposure	Control	24 h	7 days	14 days	21 days
Gill	0.16 ± 0.01	0.32 ± 0.05*	0.75 ± 0.12*	1.23 ± 0.11*	1.5 ± 0.07*
Liver	0.16 ± 0.01	0.3 ± 0.02*	0.61 ± 0.05*	1.08 ± 0.04*	1.46 ± 0.1*
Kidney	0.17 ± 0.02	0.31 ± 0.02*	0.76 ± 0.03*	1.01 ± 0.04*	1.27 ± 0.06*
Blood	0.14 ± 0.03	0.3 ± 0.02*	0.73 ± 0.08*	1.06 ± 0.08*	1.47 ± 0.05*

Values are expressed as mean ± standard deviation
*$P < 0.05$

freshwater and marine fishes have been used as models for genotoxicity testing in aquatic environments.

DNA strand breaks are measured by the comet assay which is an important biomarker of genotoxicity in fish (Mitchelmore & Chipman, 1998). Comet assay is widely used, simple, and sensitive technique for evaluating in vivo, in vitro, and in situ DNA damage in different tissues of fish such as gill, liver, kidney, and blood after exposure to different kinds of pollutants of aquatic environment (Dhawan, Bajpayee, & Parmar, 2009). The readily available and easy to collect tissue in fish is blood: 97% of total fish blood comprises of RBCs. Hence, RBCs are frequently used in fish to evaluate DNA damage by the comet assay method. Solid tissues such as gill, liver, and kidney require cell dissociation and isolation before proceeding with the comet assay procedure using such methods that themselves do not cause DNA damage. The comet assay under alkaline condition can identify DNA damage of different kinds such as single-strand breakage, alkaline labile sites, and DNA cross-links that are induced by pesticides (Kumar, Nagpure, Kwshwaha, Srivastava, & Lakra, 2010; Tice et al., 2000). Comet assay has considerable advantages over the other cytogenetic methods because in this method, the cells do not require to be mitotically dividing. (Ali et al., 2009; Ali & Kumar, 2008; Nwani et al., 2010) stated that the higher DNA damage in cells of the gills could be due to the reason that gills are constantly exposed to the pollutants of water that may cause DNA damage whereas RBCs come in contact with the pollutants only after circulation.

Most of the toxic substances that produce genotoxicity have been found to produce reactive oxygen species as well as electrophilic free-radical metabolites that interact with the DNA and lead to its disruption. Studies conducted by Chandra and Khuda-Bukhsh (2004) and Klopman, Conttreras, Reosenkranz, and Waters, (1985) showed that during the metabolism of azadirachtin, electrophilic ions and free radicals are produced, which interact with the nucleophilic sites in DNA and lead to breaks and other related DNA damage. It is also reported that the oxidative stress in an organism plays an important role to inducing cytotoxicity and genotoxicity in different vital tissues (Moore, Yedjou, & Tchounwou, 2010).

Time-dependent increase in DNA damage has been observed in the present study. The DNA damage observed in the present study can be correlated with the findings of (Cavas & Konen, 2007) on the blood cells of *Carassius auratus* exposed to glyphosate. Blood and gill cells of *Prochilodus lineatus* showed DNA damage on exposure to glyphosate (Cavalcante, Martinez, & Sofia, 2008). DNA damage in the blood cells of *Oreochromis mossambicus* increased as the heavy metal concentration increased (Ahmed et al., 2011). (Kousar & Javed, 2015) also reported DNA damage in RBC's of four fish species, showed an increase as the days of exposure to heavy metal increased. Large comet tails of DNA were seen in erythrocytes of *Cobitis elongate* on exposure to industrial effluents (Kopjar et al., 2008). Cd and Al showed genotoxic potential to zebra fish *Danio rerio* (Pereira, Cavalie,

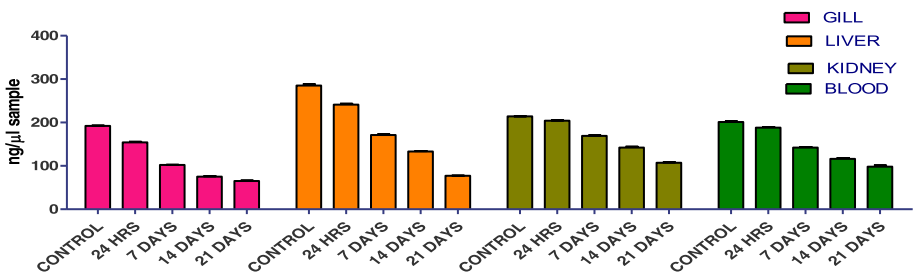

Fig. 12 DNA content in tissues of *C. carpio*, on exposure to karanjin

Table 7 Total DNA content (ng/μl) in the gill, liver, kidney, and blood in the fish *Cyprinus carpio* during exposure to sub-lethal concentration of karanjin

Tissue	Control	24 h exposure	7 days exposure	14 days exposure	21 days exposure
Gill	192 ± 0.89	154 ± 1.41*	102 ± 0.63*	75 ± 1.09*	65 ± 1.26*
Liver	285 ± 2.82	241 ± 2*	171 ± 1.61*	133 ± 1*	77 ± 0.44*
Kidney	214 ± 0.31	204 ± 0.7*	169 ± 1.3*	142 ± 2*	107 ± 1*
Blood	201 ± 1.43	188 ± 1.41*	142 ± 1.41*	116 ± 2.28*	98 ± 2.82*

Values are expressed as mean ± standard deviation
*$P \leq 0.05$

Camilleri, Gilbin, & Adam-Guillermin, 2013). Results observed in the present study can be compared with those of (Kousar & Javed, 2014) where red blood cells of carps showed significant ($P < 0.05$) DNA damage due to heavy metals. A study performed by Matsumoto et al. (2006) showed significant increase in DNA damage in the peripheral blood erythrocytes of *Oreochromis niloticus* exposed to chromium. The results of the present studies are in agreement with the finding of Kumar et al. (2010) which proved that malathion can cause DNA damage in *Channa punctatus*. Common carp when exposed for 3 days to a textile dye effluent showed DNA damage, which was measured using comet assay (Sumathi, Kalaiselvi, Palanviel, & Rajaguru, 2001).

Nucleic acid content of an organism is considered as an index of capacity of protein synthesis. The development and growth of the fishes depend upon the content of DNA which serve as important biochemical parameter (Buckley, 1980). Active protein synthesis and cell growth are dependent on the DNA content. Whenever an organism is exposed to pesticide-dissolved water, its nucleic acid content changes. (Ansari & Kumar, 1988) have reported a significant decrease in the DNA content of liver of zebra fish, *Danio rerio*, on exposure to cypermethrin. A significant decline in the DNA content in *Labeo rohita* on exposure to ammonia was observed by Acharya, Dutta, and Das (2005). Maruthanayagam and Sharmila (2004) conducted studies on the effect of monocrotophos on *Cyprinus carpio* and concluded that the pesticide leads to several changes in DNA which may be due to the increased activity of the enzyme DNase function.

Significant reduction in DNA content in different organs of fish exposed to karanjin in the present investigation may be due to decrease in protein synthesis, defective nucleic acid metabolism, and also degradation of cells. Another important reason for DNA damage may be the inhibition of enzymes that replicate or repair DNA (Singh et al., 1988; Aruoma, Halliwell, Gajewski, & Dizdaroglu, 1991; Guilherme, Santos, Barroso, Gaivao, & Pacheo, 2012). The intactness of the DNA is the important part of the normal cellular process. The changes in DNA may be due to loss of cell structure, proliferation of new cells, formation of new tissue, and tissue degradation (Youson, 1988) which ultimately results in total loss of cellular control mechanism (Heath, 1998). Many pesticides have been proven to induce changes in DNA (Massimo, Milena, Scassellati, & Rossana, 2000; Vrhovae & Zeljezic, 2000) and structural changes at the level of chromosomes (Mathur, 1988). The physiochemical interaction of the pesticides with the cellular DNA produces a number of primary changes such as single-strand breaks, double-strand breaks, DNA protein cross-link, and damage to purine and pyrimidine bases (Van Loon, Groenendijk, Van Der Shcanslohman, & Bran, 1991). Similar results were also reported by various

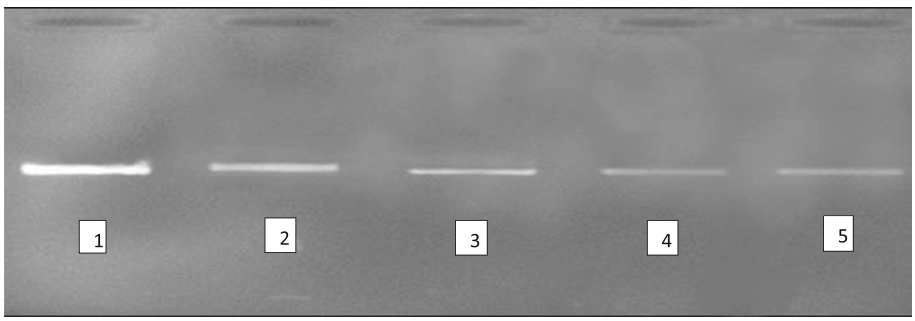

Fig. 13 Gel image showing DNA of the gill

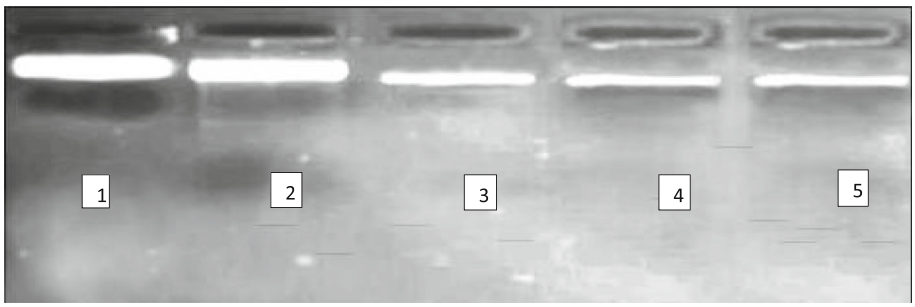

Fig. 14 Gel image showing DNA of the liver

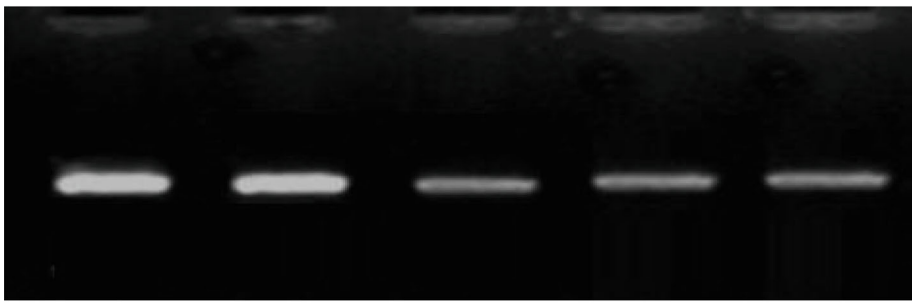

Fig. 15 Gel image showing DNA of the kidney

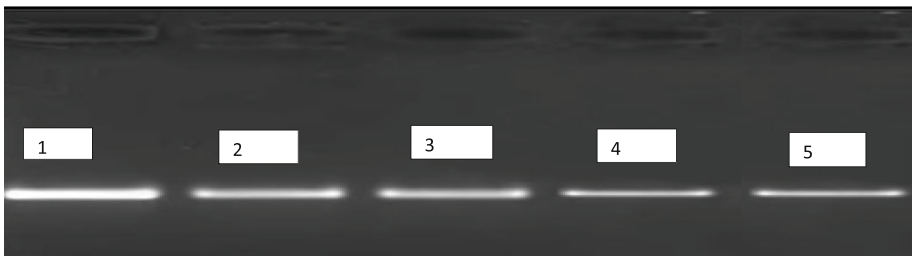

Fig. 16 Gel image showing DNA of the blood

investigations: *Cyprinus carpio* (Gowri, Govindassamy, & Ramalingam, 2013), *Cirrhinus mrigala* (Veeraiah, Vivek, Srinivas Rao, & Venkatrao, 2013), *Channa punctatus* (Thakur & Kakde, 2012), *Labeo rohita* (Tiwari, Tiwari, & Singh, 2012), *Colisa lalia* (Singh, Singh, & Yadav, 2010), *Cirrhinus mrigala* (Vasantharaja, Pugazhendy, Meenaambai, Prabukaran, & Dar, 2013), *Channa striatus* (Raksheskar, 2012), *Channa punctatus* (Kumar, Sharma, & Pandey, 2007), and *Cyprinus carpio* (Ansari & Kumar, 1988). *Channa punctatus* is exposed to methanol (Tiwari & Singh, 2003).

Conclusion

In conclusion, the results of the present study indicate that the comet assay or alkaline SCGE is a very sensitive test showing clear response of an organ or tissue towards dose and exposure period. Comet assay has many advantages over other types of commonly used assays in that it can be used to assess the genotoxicity in a wide range of organisms, in different types of tissues. Karanjin, though a plant secondary metabolite derived from plant, caused genotoxicity in the common carp *Cyprinus carpio* during sub-lethal exposure, and also, the total DNA content in gill, liver, kidney, and blood decreased as the days of exposure increased. Hence, comet assay or SCGE is preliminary and the best assay to assess genotoxicity in any organism and any kind of tissue.

Abbreviations

%: Percentage; B: Blood; DNA: Deoxyribose nucleic acid; EDTA: Ethylenediaminetetraacetic acid; EtBr: Ethidium bromide; G: Gill; K: Kidney; L: Liver; LC: Lethal concentration; LM Agarose: Low-melting agarose; SCGE: Single-cell gel electrophoresis; UV: Ultraviolet

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Competing of interests

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Availability of data and materials

All the data and materials presented in the manuscript are the original work of the authors.

Authors' contributions

ST designed the work and performed the experiment. ST and RY have written the manuscript. Both the authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the guidelines of IAEC (Institutional Animal Ethical Committee), and approval was taken in order to perform the experiments.

Consent for publication

We give our consent to The Editor of The Journal of Basic and Applied Zoology (Springer) that our manuscript be published in their journal.

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