Evaluation of Acute Toxicity and Genotoxicity Of DiButyl Phthalate(DBP) On Fresh Water Cyprinid Fish Comman Carp (*Cyprinus Carpio* .)

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***Abstract*-The present study aimed at determining the acute toxicity (LC_{50} Value), and genotoxicity of dibutyl phthalate (DBP) on *Cyprinus carpio.* The acute toxicity assay was carried out according to the standard methods in APHA and the value was assessed using the Probit Analysis method. The result confirmed that the median lethal dose (LC_{50}) of DBP for the fish, *C. carpio* is 7.76 ppm. Signs of abnormal behavior were also noticed during the test such as loss of equilibrium, erratic swimming, lethargy and motionlessness. The study concluded that DBP is highly toxic to fish, *C. carpio* with the evidence of behavioral deformations. For genotoxicity evaluation, fishes were exposed to sublethal ($1/_2$ LC_{50} -3.86 ppm) for 96 hrs. After the completion of exposure period, fishes were anesthetized and cardiac puncture was done. The comet assay was performed and the statistical analysis revealed significant ($p<0.05$) DNA damage in DBP treated group. The study demonstrated the utility of comet assay for in vivo laboratory studies using fish for screening the genotoxic potential of DBP.**

***Index Terms*--Acute toxicity, *C.carpio,* Comet assay, DBP, Genotoxicity, , LC_{50}.**

• INTRODUCTION

Fishes have significant economic importance and are quite sensitive to the wide array of pollutants discharged in the aquatic ecosystems. Fishes are widely used to evaluate water standard of aquatic environment because they serve as pollution bioindicators and play notable roles in assessing potential risk associated with contamination of new chemicals in aquatic ecosystem[1]. In Kashmir Cyprinids are the most notable family of fish, and its members are distributed globally. These family members are distributed broadly in fresh water sources [2], [3] Freshwater Cyprinid fish dominates global aquaculture production. Some characteristics of *C. carpio*. (Cyprinidae) such as its wide distribution and availability throughout the year, cost-effectiveness, easy handling and acclimatization in the laboratory make it an excellent ecotoxicological model.

Environmental pollutants, like xenobiotic substances released as byproducts of anthropogenic actions, naturally lead to pollution of aquatic environments. They negatively affect the environment through

unfavourable impacts on growth, development and reproduction of aquatic corganisms [4], lead to a keen fall in number besides quality of the aquatic population [5]. As a downstream impact, such pollution also affects human and animal health chiefly in cases where fish is consumed or utilized as a food source. This is because fish are common pollutant bioaccumulators and have the highest potency for transferring such residues to humans [6]. One of the outstanding examples of xenobiotics is endocrine disrupting compounds (EDCs) such as phthalate esters (PEs), which have the efficacy to disturb numerous biological systems including the invertebrate, reptilian, avian, aquatic and also the mammalian systems [7].

Phthalates are family of xenobiotic hazardous compounds amalgamating in plastics to intensify their plasticity, flexibility, longevity, versality and durability. Besides they are also used as lubricants, solvents, additives, softeners etc. They are present in number of day to day used products such as PVC products, building materials (paint, adhesive, wall covering), personal-care products (perfume, eye shadow, moisturizer, deodorizer etc), packaging, children's toys, pharmaceuticals and food products, household applications such as shower curtains, floor tiles, wrappers etc. They are ubiquitous environmental contaminants entering environment via various routes. Once entering the environment, they pose remarkable toxicological threats to the myriad of non target organisms, discover its way to the food chain, and threaten ecological balance and biodiversity of nature. The effluents generated from waste water treatment plants have been considered as main source of plasticizers in aquatic environment [8]. Due to potential risk of phthalates for organism's health and environment, a number of them have been incorporated in the priority pollutant list of several national and supranational federations.

Dibutyl phthalate(DBP) is one of the commonly used phthalate essentially as plasticizer to ameliorate the flexibility and workability of the products, such as polyvinyl chloride, plastic packaging films, adhesives, cosmetics and insecticides [9]. DBP is not chemically attached to the polymer matrix like other phthalates, directing to its ubiquitous existence in the diverse environmental matrices [10]. DnBP is considered very dangerous substances in the EU REACH regulation and is classified as category 1B in the Commission Directive 2007/19/EC (cannot be used to make toys, childcare articles, and cosmetics) and risk reduction measures are required for its safe use. Canada and the United States have also taken regulatory actions restricting their use [11]. Furthermore, it poses a particular risk to aquaculture.

Toxicity tests have been performed on fishes to estimate the effect of toxins on various aquatic organisms under laboratory conditions. The 96-h acute toxicity, described as median lethal concentration (LC_{50}) value is contemplated appropriate for toxicological testing and safety assessment of the organic chemicals. The LC_{50} value of a chemical is defined as its concentration in water that kills 50% batch of test animal (fish in this study) within a continuous period of exposure which must be stated.

The importance of evaluation of genotoxicity of various pollutants including Phthalates in fish lies in the fact that higher vertebrates, including humans feeding on fish, are conveniently exposed to the genotoxic agents that are trapped in fish body. Since there is increasing concern over the existence of genotoxins in the aquatic environment. It is significant to develop or systematize the already existing methods for determination of genotoxic chemicals in aquatic organisms [12]. Establishment of most economical and sensitive technique under alkaline state for the identification of genetic damage at cellular level, The Comet assay having sensitivity for identifying minimum intensity of DNA fragmentation and necessitate a small number of blood cells per fish specimen [13], [14]. The principal advantage of Comet assay are the small sample size requirement, its swiftness and the probability to distinguish between cell types concerning the degree of DNA damage or DNA repair level.

II. MATERIALS

The chemicals used in the current study were of high clarity. Di-n-butyl phthalate ($C_{16}H_{22}O_4$, DnBP, CAS No. 84-74-2, 99% purity) was procured from Sigma- Aldrich; Bengaluru, India is a colorless to faint yellow viscous liquid. Acetone, $(CH_3)_2C0$, CAS No.67-64-5, 99% purity was purchased from Hi- Media Labs, Mumbai, India. Agarose-normal melting (molecular biology grade-MB), Agarose-low melting (MB), sodium chloride (analytical reagent grade-AR), potassium chloride (AR), disodium hydrogen phosphate (AR), potassium dihydrogen phosphate (AR), di sodium EDTA(AR), Tris base (AR), sodium hydroxide (AR), sodium lauryl sarcosinate (AR), triton-100 (MB), Hydrochloric acid (AR), Ethanol, Ethidium bromide(MB) and benzocaine (AR), formalin , paraffin wax, hematoxylin , eosin etc were purchased from himedia and are 99.95% of purity.

III. METHODS

A.Test Organism

C. carpio. (Family: Cyprinidae and Order: Cypriniformes was selected as the experimental model. It is a freshwater fish occurring in the standing and slow flowing waters, especially the flat land lakes of the Kashmir Valley. Live juvenile fish were procured with the help of a local fisherman, using hand nets, from the Dal Lake (34°07'N 74°52'E), in the vicinity of the University of Kashmir, Srinagar, India. They were transported live in plastic jars to the Cytogenetics and Molecular Biology Laboratory, Centre of Research for Development (CORD), University of Kashmir and subjected to a prophylactic treatment by bathing in a 0.05 % aqueous solution of potassium permanganate for 2 m to avoid dermal infection. Their average length and wet weight (± SD) were recorded as 20.5 ± 1.64 cm and 33 ± 4.94 g, respectively.

B. Acclimatization

The fish stock was acclimatized before the commencement of the experiment for at least 3 weeks to a 1:1 diurnal photoperiod in well aerated 60 L glass aquaria at 19.7 ± 2.6°C with 24 h aged dechlorinated tap water (pH 7.6 – 8.4) and fed daily with commercially available fish food (Feed Royal®, Maa Agro Foods, Visakhapatnam, Andhra Pradesh, India). Only active specimen with no sign of injury and distress were used in the study. Waste products were siphoned off every day to check increase of ammonia in the water. Every effort as suggested by Bennett and Dooley [19] was taken to maintain optimal conditions during acclimatization: no fish died during this period. The acclimatized fish were used for the experiments. Studies involving experimental animals were conducted in accordance with the guidelines described for maintenance, care and conducting toxicity tests of fish in Standard Methods for the Examination of Water and Wastewater, American Public Health Association [20].

C. Median Lethal Concentration Assessment (96-h LC_{50}) And Toxicity Symptoms Of DBP In Juvenile Fish

Determination of 96 h- LC_{50} of DBP to *C. carpio* was conducted in a semi-static system with 60 L glass aquaria, changing the DBP solution after every 24 h to maintain similar concentration throughout the experiment. Following range finding tests, fishes were exposed to eleven different concentrations of DBP (2,4,6,8,10,12,14,16,18,20,22 ppm). Each group was assayed in duplicate. Blank controls one of tap water and another of acetone were also included in experiment. Each test group was exposed to varying concentrations of DBP and the resultant mortalities were counted and recorded at 24, 48, 72 and 96 h intervals. No food was provided to the fish throughout the experiment and lethality was the toxicity end-point. Fish were visually examined daily and considered dead when no respiratory movements or no sudden swimming in response to gentle touching were observed. Dead fish were gently and immediately removed from the aquaria. During the acute toxicity testing, fishes were examined for abnormal behaviour and external appearance. Finally to find out the concentration of DBP at which 50% mortality of fish could occur, the Probit Statistical Analysis [21] was done using SPSS statistical analysis software (24.0) and LC_{50} was determined with (95% confidence limit). Moreover to make analysis conducive regression equation (y=mortality and X=concentration) was found out, the LC_{50} was derived from the best-fit line obtained.

D. Genotoxicity Testing Using Comet assay

Exposure conditions

Acclimatized fish specimen were subjected to sublethal concentration (1/2 LC_{50}) of DnBP for the period of 4 days. Following standardized OECD testing guidelines 40 fishes were dived into 4 groups comprising of 10 fish each. Group I (negative control) fish were given no treatment, Group II was solvent group (Acetone), Group III fish were exposed to sub lethal DnBP concentration (3.86ppm) and group IV fish were (positive control)

exposed to 5ppm of ethyl methanesulphonate (EMS). Aquaria were continually aerated during the experiment and medium were renewed on daily basis and fresh solutions were spiked to maintain water quality with DnBP level. During exposure period, fishes were examined for abnormal behaviour and external appearance.

Comet assay/Single cell gel electrophoresis (SCGE)

The comet assay was undertaken according to Singh et al protocol [13]. The single cell gel electrophoresis (SCGE)/ comet assay, evolved by N.P. Singh, merges the simpleness of biochemical procedures for investigating DNA single strand breaks incomplete excision repair sites, frank strand breaks, alkali-labile sites and cross linking with the single cell perspective representative of cytogenetic assays.

Prior to blood collection, fish were anaesthetized with 0.12 mg/L benzocaine. Cardiac puncture was done and 0.3mL of blood was drawn. 5µL of blood sample was diluted in 1000µL of PBS. Cell viability was assessed by Trypan Blue Exclusion technique and the blood samples showing 84% cell viability were processed for the Comet assay.10µL of cell suspension mixed with 120µL of 0.5% low melting point Agarose (LMPA) at 37%, were layered on previously 1% normal melting pointing Agarose (NMPA) coated slides. The slides were carefully cover-slipped and placed in refrigerator at 4˚C for 10-15 min, for solidification of the gel. Once the blood-LMPA layer was solidified, cover slip was carefully removed avoiding avulsion of the underlying layer. 75µL of LMPA was added onto the Agarose gel mixture layer and a new cover slip was placed carefully over the gel mixture layer, gel was allowed to solidify at 4˚C in a refrigerator for 10-15 min. After solidification of gel, cover slips were carefully removed and slides were immersed into freshly prepared cold lysing solution and refrigerated for atleast 1hr at 4˚C. After lysing, the slides were benignly removed from lysis solution and placed exactly perpendicular to both the electrodes with the Agarose-coated side facing upwards in horizontal submarine gel electrophoresis system. The electrophoresis tank was filled with cold fresh electrophoresis buffer till the buffer completely covered the slides without formation of air bubbles over the Agarose gel. The slides were kept in alkaline buffer for 30 min to allow the unwinding of DNA strands and expose the alkali labile sites (alkali unwinding). Power supply was turned on with 0.74V/cm (between electrodes) and current was adjusted to 300mA by raising and lowering the buffer level. Electrophoresis was carried out for 30min. After 30 min of electrophoresis, power supply was turned off and slides were gently lifted from electrophoresis buffer and were placed on a drain tray. Drop wise slides were coated with neutralization buffer for 5min and the buffer was drained; the process was repeated two more times followed by numerous was with distilled water. After draining, slides were immersed in 100% ethanol for for dehydration. Slides were air dried and placed in an oven for 50min .The slides were then stored at dry area. For staining, slides were rehydrated with chilled distilled water for 30 min and were stained with 50µL of Ethidium bromide and cover slipped. Before viewing excess stain was blotted away from back and edges. For visualization of

Ethidium bromide–stained slides, fluorescent microscope equipped with an excitation filter of 515-560nm with barrier filter of 590nm and a magnification of 200X was used.

Visualization And Comet Scoring

For the assessment of DNA damage, images of 300 randomly selected cells (100 per slide) were analyzed for each sample. The cells were scored visually according to tail length into five categories, from undamaged class (0) to complete damaged class (type IV)[22]. The data are presented as the frequency of cells with and without DNA damage, score and distribution of classes. The calculation of score was done by multiplying the number of nuclei found in a class times the class number. Statistical analysis was performed with student's t- test. Analysis was carried out using SPSS (version 24.0) for windows considering a significance level of $p<0.05$.

IV. Experiments and Results

- ***LC_{50} Value And Clinical Observations***

The calculated 96-h LC_{50} value (with 95% confidence limit) for DnBP, using a semi static bioassay system on *C. carpio* was found to be 7.76 mg/L as shown in Table I. The relation between percent mortality rate and the concentration of DnBP have been drawn according to Finney's Probit analysis using SPSS statistical analysis software (24.0). Fig.1 shows the regression line between the mortality of *C. carpio* and log concentration of DnBP. It was observed that a dose-dependent increase and time –dependent decrease occurs in mortality rate such that as the exposure time increases from 24h to 96h, the median lethal concentration required for killing the fish was reduced. No mortality was observed in control groups during whole experiment

Table I

96h-LC_{50} Value of DBP to *C.carpio.*

Compound	LC_{50} (mg/L)	Regression equation	Correlation coefficient (R^2)	Standard Error
DBP	7.76	Y =2.682x + 2.613	O.95	0.21

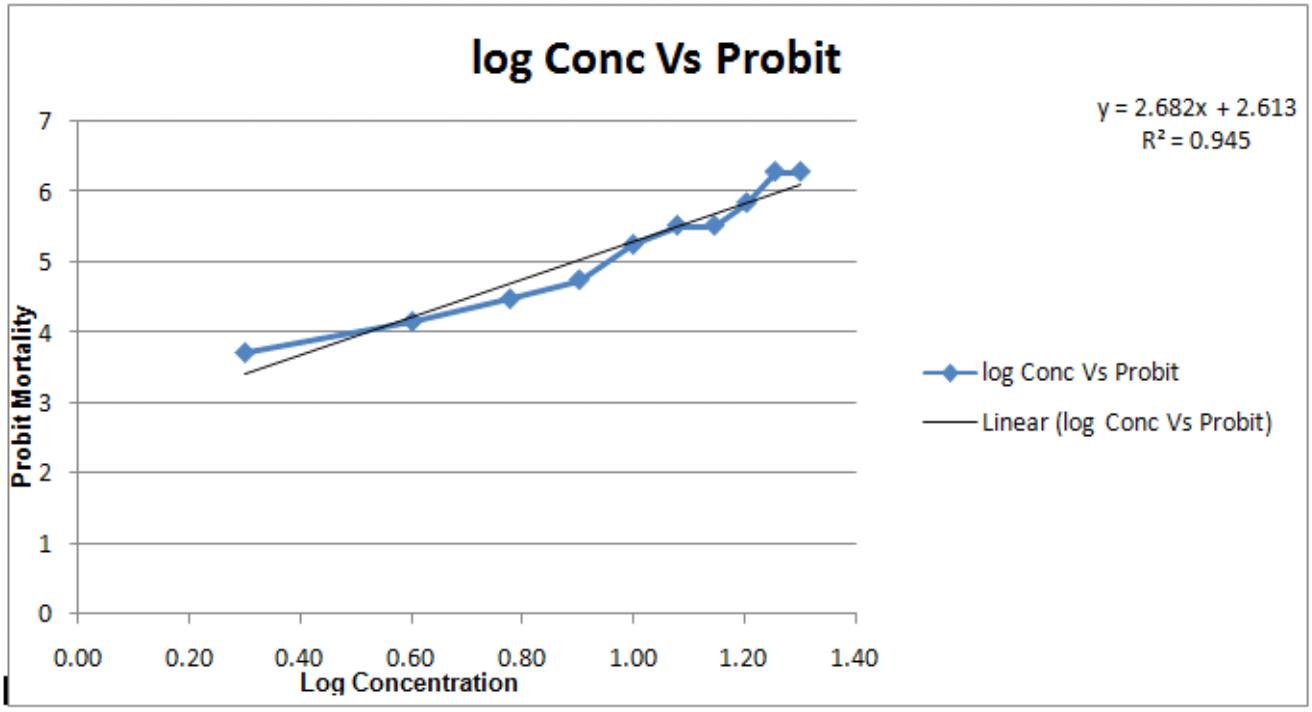

Fig. 1. Regression line showing positive correlation between probit mortality of *C.carpio* and log concentration of DnBP.

During acute toxicity assay no clinical signs were noticed throughout the period of exposure in control groups. However, within 8h of exposure to DnBP, Carps in each group showed different intoxications symptoms. In the higher concentration groups, fishes were fully evoked shortly after contacting the solution contrast to lower concentration groups, but the intoxication characters were same as in higher concentration groups. Abnormal behaviour was noted immediately such as loss of equilibrium, erratic swimming movements, lethargy and motionlessness followed by convulsions.The fish under experimental study exhibited difficulty in breathing represented by speedy breathing coexisting with rapid movement of operculum and failure to respond to escape reflex. Furthermore dark discoloration of skin with thick layer of mucous was also noted. Postmortem studies revealed congestion of internal organs and excessive slime deposition on gills.

.Dna Damage Analysis

Analysis of number of cells with comets in the blood samples demonstrated that there was a significant DNA damage in DBP treated group (1/2 LC_{50} -3.88ppm) compare to all 3 control groups; positive control (5ppm EMS), negative control group (untreated) and solvent control group (acetone). There was approximately 10 times the number of cells with DNA damage in treated group comparing to the negative and solvent control groups. However, comparing positive control group, the treated group showed borderline significance in DNA damage level. Fig. 1 shows the different classes of comets formed in DnBP treated group.

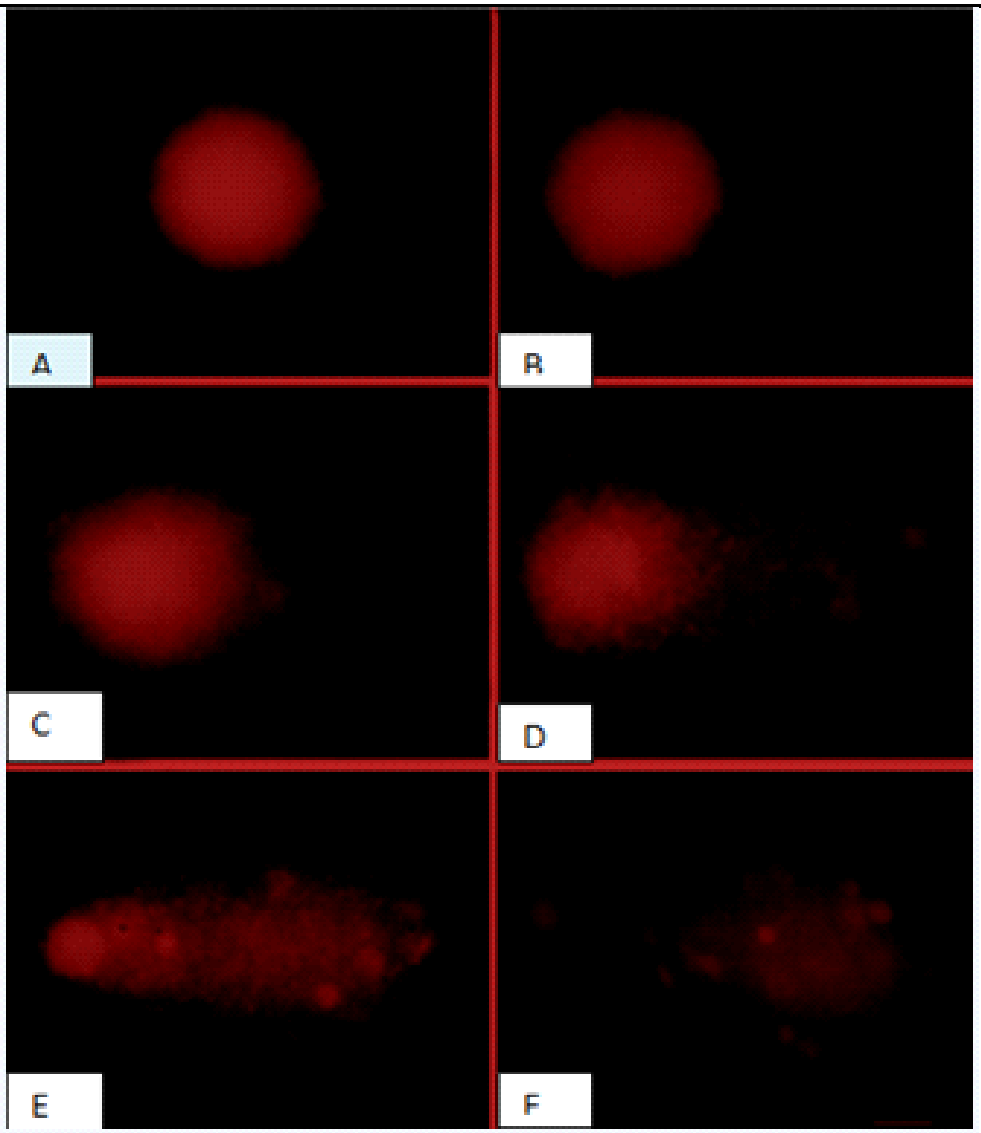

Fig. 1. Photomicrographs showing the classification of blood cells ,treated with sublethal DBP (3.88ppm), in the comet assay. A, Class 0, undamaged; B, class 1; C, class 2; D, class 3; E, class 4, maximum damage; F, apoptotic cell.

The mean score, which indicates the degree of damage in the analysis of the blood cells, found in the treated group (μ= 282) was little higher than that of positive control (μ=247.3). However the mean score in treated group was approximately about 10 times the value determined in the negative control (μ=25) and solvent control(μ=24.6). Statistical analysis demonstrated that the mean score of treated group was significantly greater ($p<0.05$) when comparing to all three control groups.

Discussion

At present, numerous chemicals have been classified as plasticizers and studies using different models have indicated that some of them have toxic properties. Pollution of aquatic environment due to plastic residues is well documented and fish are often used as sentinel organisms for eco-toxicological studies as they are able to accumulate genotoxic substances and respond to low concentration of mutagens in a manner similar to higher

vertebrates [24], [25]. Therefore, the use of fish biomarkers as indices of the effects of pollution, are of great importance, and help in early detection of aquatic environmental problems [15].

In the present study, pre-treatment of (0.05 %) solution of potassium permanganate was given to the fish for 2 min to avoid any dermal infection and after that the specimen were acclimatized for at least 3 weeks under laboratory conditions to remove the residual effects of other chemicals prior to start of the experiment. Several investigators [12], [26], [27] have used potassium permanganate solution for prophylactic treatment before starting their experiments, and like our study, they did not report any adverse effects in the test organism due to prophylactic treatment.

A number of studies have been published on DBP toxicity in early stages of aquatic species [28], [29] but reports with regard to its toxicity using LC_{50} fractions are relatively sparse. In an attempt to fill this lacuna, the study was conducted to assess the 96h- LC_{50} of DBP in *C.carassius* using Probit Analysis. The Probit analysis is commonly used in toxicological studies to determine relative toxicity of chemicals to living organisms. In present study probit analysis has been done by drawing regression line between probit kill of *C.carpio* and log of concentration of DBP. Finally, the calculated 96h- LC_{50} of DnBP for *C.carpio*, as obtained based on Probit Analysis, was 7.77mg/L. The subsequent data indicated that DnBP at the given concentration is highly toxic to *C.carrassius*. Several acute toxicity studies have been reported for 96 h -LC_{50} values in case of fish species other than *C.carassius*; the reported 96h-LC_{50} value of DBP in Nile tilapia (*Oreochromis niloticus)* was 11.8mg/L[30] Also, in a study, Zhao [31] reported that the DEHP 96-h LC_{50} in case of carp (*Cyprinus carpio*) is 16.30 mg/L. The noticed variation in the sensitivity of fish to DBP can be accounted for by differences in kinetic parameters, species, size, age, health as well as experimental conditions [32]. The valuable scientific data drawn from acute toxicity studies was acquired from a combination of behavioral, clinical and postmortem observation of test animals in addition to the LC_{50} value [32]. The clinical alterations observed in the test subjects exhibited as perturbations in their respiratory and movement patterns and seemed to appear almost immediately after exposure to high DBP concentrations, where these behavioral deviations became more pronounced as DBP levels were increased. The altered respiratory pattern may be a byproduct of post-stress related excessive mucus secretion which results in the formation of a thick coat on the gill tissue which causes irritation to the gills Behavior-related alterations observed in our study are hypothesized to be a strategy by which the animals adapt to changes in the surrounding environment upon exposure to pollutants. The study on fishes is of great importance to provide a future understanding of ecological impact.

The genotoxic impact of environmental contaminants can be tracked using wide variety of both Invitro and invivo biomarker assays but the comet assay is getting more popularity over the other assays due to its

advantages like sensitivity in detecting low levels of DNA damage (0.1 DNA break per 10^9 DA) [33], completion of assay in short time, low number of cells required, cost effectiveness, precision, ease of application, The cells used in this assay do not need to be mitotically active, requires neither metaphases nor knowledge of chromosome number [14], [34]-[37]. Accordingly, it has been broadly used in the fields of genotoxicology and environmental biomonitoring [14], [38] inclusive of aquatic organisms [39] as potent tool to measure the relationship between DNA damage and the exposure of aquatic organisms to genotoxic contaminants. The alkaline comet assay [13] is able to identify DNA damage, i.e. single strand breaks or other lesions, such as alkali labile sites, DNA cross-links [40] and incomplete excision repair events [33]. Strand breakage level in DNA has been suggested as a sensitive indicator of genotoxicity and as effectual biomarker in biomonitoring of the environment[41].

In our study, within the same group blood cells were distributed in different degrees of DNA damage to indicate that comet assay was capable to detect intercellular differences in DNA damage of heterogeneous mixture of cells. The distribution of the DNA damage grades in all the DBP treated groups were significant then the control ($p<0.05$). Though there is sparse data on the genotoxic potential of DBP on fishes, our study shows relevance with the studies conducted by other authors. A study done on Nile tilapia (*oreachromis niloticus*) to find the genotoxic impact of di-n-butyl phthalate on juvenile by using Alkaline comet assay [30]. The studies confirmed that there was significant DNA damage in fish exposed to DBP. Another study demonstrated the DNA damaging potency of chronic exposure of DBP on Nile tilapia fingerlings using comet assay. The result showed significant increase in DNA damage. The study concluded comet assay as sensitive tool detecting lower levels of DNA damage [42].

The pervasiveness of genotoxic contaminants in the aquatic ecosystems is one of the crucial concern in the field of environmental science and this has demanded the need to evolve sensitive techniques to monitor the genotoxic potency of these chemicals in aquatic organisms[43]. Fishes are astonishing model organisms for Genotoxicological studies and furnish prior warnings for toxicants induced environmental degradations and alterations [44] including evaluation of contaminants in aquatic ecosysytems [45] . The SCGE /Comet assay is being broadly used as a biomarker for the identification of genotoxic impacts of chemicals in aquatic organisms [12], [46]-[49]including detection of DNA damages propitiously in fish [50],[51]

VI. Conclusion

The study on fishes is of great importance to provide a future understanding of ecological impact. The present study was an attempt to find the acute toxicity, genotoxicity and histopathological impact of DBP on *C.carpio.* The results of acute toxicity exclusively showed that administration of DBP induced mortalities in model organism *C.carpio* at various concentrations confirming its acute toxic potential. The median lethal concentration (96 h-LC_{50}) of DBP was 7.76mg/L, thus confirms that it belongs to high toxic level compounds. The current study also showed the considerable advantage of comet assay over the other cytogenetic assays to detect DNA damage like chromosome aberrations, sister chromatid exchange and the micronucleus test, because to carryout comet assay , the cells don't need to be mitotically active, On the basis of experimental evidence obtained, we suggest that DNA damage in blood cells of freshwater cyprinid, *C.carassius* may potentially be used as bioindicators for detecting the genotoxic nature of DBP. The results of histopathological examination of gills and liver after sublethal exposure to DBP showed alterations in both tissues. Therefore, we suggest that the histopathological changes of certain target tissues acts as biomarkers of environmental exposure of freshwater fish to DBP. Additional studies are needed to shed light on chronic exposure of DBP and evidence of repair during period following a DBP exposure.

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