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Protective efficacy of biogenically synthesized glucose-capped ZnO nanoparticles against Bisphenol A in *Channa punctatus* (Bloch, 1973) in the term of Micronuclei induction in RBCs

Amisha Verma* and Vivek Kumar
Department of Zoology, Isabella Thoburn College Lucknow – 226007
Email address: vermaamisha900@gmail.com

Abstract

India's fast urbanization and industrialization have led to significant freshwater contamination, especially from endocrine-disrupting chemicals (EDCs) including Bisphenol A (BPA), a synthetic estrogen frequently detected in wastewater. It has been shown that Bisphenol A negatively affects aquatic organisms' endocrine systems. The current study uses the cytogenetic end point micronuclei test to screen for the protective impact of *Morus nigra* aqueous leaf extract (G-ZNPs), which may lessen the harmful effects of BPA in freshwater fish *Channa punctatus*. A simple and reliable technique for evaluating the genotoxic properties of various drugs in vivo is the micronucleus test. This approach was utilized in genotoxicity and mutagenesis to investigate possible genotoxic risk resulting from exposure to dangerous xenobiotics in a variety of animals, especially aquatic sensitive organisms. In the first series of experiments, fish were given regulated and glucose-capped ZnO nanoparticles (3 mg/l) in addition to a single dose of Bisphenol A for 96 hours ($LC_{50}/10$). Three concentrations of *Morus nigra* aqueous leaf extract (G-ZNPs) (1 mg, 2 mg, and 3 mg) were run concurrently with the same concentration of Bisphenol A ($LC_{50}/10$) in the second set of experiments. The incidence of micronuclei induction was considerably decreased in a combination treatment with *Morus nigra* aqueous leaf extract. The results indicated that the aqueous leaf extract of *Morus nigra* (G-ZNPs) showed antigenotoxic action in this fish model under the current experimental conditions.

Keywords: Bisphenol A, *Channa punctatus*, Genotoxicity, Micronuclei test, *Morus nigra*

1. INTRODUCTION

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl) propane]) is a carbon-based synthetic organic compound belonging to the class of phenols. In the presence of a catalyst, phenol and acetone condense under acidic circumstances to produce bisphenol A. The International Union of Pure and Applied Chemistry (IUPAC) refers to it as 4,4'-dihydroxy-2,2-diphenylpropane, (European Commission Joint Research Centre, 2010) It has the chemical formula $C_{15}H_{16}O_2$ and a

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molecular mass of 228.29 g.mol-1. It is less soluble in water and more soluble in ethanol, acetic acid, and diethyl ether (Cao, 2011).Bisphenol A is a crucial component in the manufacturing of thermal printer paper (Biedermann et al., 2010) and polycarbonates, epoxy resins, and polyester resins (Vandenberg et al., 2007).They are utilized, among other things, in the production of toys for infants and children, lenses for glasses, packaging media, compact discs, and window panels, as well as products that come into contact with food (reusable bottles, including baby bottles, containers for beverages and foods) (Vandenberg et al., 2007; Staples et al., 1998; Dekant & Völkel, 2008; Huang et al., 2012).

Humans are exposed to BPA through ingestion, inhalation, or skin contact once these products are discharged into the environment (Metz, 2016). Even though BPA has a short half-life of 1–10 days in soil, it is nonetheless a significant pollutant due to its widespread use (Huang et al., 2012). As a result, exposure to BPA can occur through the air, water, and soil. BPA exposure has been linked to an increased risk of carcinogenesis and an increase in the susceptibility of specific cell types (Porreca et al., 2016). Additionally, BPA has recently been demonstrated to directly or indirectly cause genotoxicity in a variety of in vitro systems (Jalal et al., 2018).

Aquatic ecosystems are continuously exposed to BPA through landfill leachates, urban sewage, and petrochemical industry discharges; this poses a major threat to aquatic organism health (Flint et al., 2012; Rubin, 2011). BPA can also be released into the water by plastic waste. Its detrimental effects on aquatic animals have raised serious concerns (Buxton & Kolpin, 2005; Huang et al., 2012) since its toxicity can affect fish populations, behavior, and central nervous systems (Hayashi et al., 2015). Furthermore, fish growth, morphology, biochemical variables, and histological structure can all be greatly impacted by BPA (Pastva et al., 2001; Lam et al., 2011; Kinch et al., 2015; Hamed & Abdel-Tawwab, 2017; Yaghoobi et al., 2017). BPA negatively affects aquatic species' growth at every stage, according to a comprehensive meta-analysis on the subject (Wu & Seebacher, 2020). The numerous effects of BPA on aquatic animals, such as fish, amphibians, aquatic reptiles, and mammals, have been thoroughly examined by researchers (Franzellitti et al., 2019).

According to Monteiro et al. (2010), fish have been regarded as an excellent and efficient model for assessing the toxic, mutagenic, and carcinogenic potential of contaminants. They are the most extreme occupants of all aquatic habitat zones, bioaccumulate environmental toxins, react to mutagens at low concentrations, and offer early warning of changes in the environment

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brought on by pollution. India and its neighboring countries are home to the freshwater fish Channa punctatus (Bloch, 1793). It favors dirty, stagnant water to flowing water. It is carnivorous and consumes worms, fish fry, and small fish. It is a common edible fish with nice meat. It is affordable, resilient, and simple to maintain in lab settings (Yadav and Trivedi, 2006). There are over fifty species in the genus *Channa* that have been described by science.

The Moraceae family includes the Black Mulberry (*Morus nigra*), which grows in East, West, and South-East Asia, South Europe, South of North America, Northwest of South America, and some parts of Africa (Calín-Sánchez et al., 2013) in addition to Turkey (Boschini, 2002). More than 80,000 tons of mulberries are produced annually (Ercişli & Orhan, 2007). Many nations employ black mulberry fruit, leaves, and even branches as blood pressure-lowering medications, diuretics, and antipyretics (Zhishen et al., 1999). The literature has extensively examined the primary bioactive substances found in Black Mulberry fruit. Flavonoids, anthocyanins, phenolic acids, and carotenoids are among the many phenolic chemicals found in it (Lin and Tang, 2007; Du et al., 2008; Özgen et al., 2009; Kutlu et al., 2011; Fazaeli et al., 2012; Calín-Sánchez et al., 2013). According to Bae and Suh (2007), black mulberry's potent bioactive ingredients are anthocyanins. The levels of phenolic compounds, vitamin C (ascorbic acid), and overall antioxidant capacity were thoroughly examined by Gundogdu et al. (2011). Green produced zinc oxide nanoparticles have drawn a lot of interest because of their strong biological effects, biocompatibility, and environmentally benign manufacture. Plant extracts like *Morus nigra* function as stabilizing and reducing agents in green synthesis, enabling the creation of nanoparticles without the use of hazardous chemicals. Phytochemicals including flavonoids and phenolics, which are abundant in these biosynthesized ZnO nanoparticles, improve their antioxidant qualities. According to studies, ZnO nanoparticles may efficiently scavenge reactive oxygen species (ROS), which lowers oxidative stress brought on by environmental pollutants like bisphenol A. This decrease in oxidative stress aids in shielding exposed organisms against chromosomal abnormalities, DNA damage, and the development of micronuclei.

The micronucleus test is a straightforward and accurate method for assessing the genotoxic qualities of different substances in vivo. As "small particles consisting of acentric fragments of chromosomes or entire chromosomes which lag behind at anaphase stage of cell division," micronuclei can be described. These pieces might not be found in the offspring cells' nucleus

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during telophase and instead form one or more micronuclei in the cytoplasm (OECD, 1997). A popular cytogenetic technique for evaluating chromosomal damage brought on by different genotoxicant is the micronucleus test (MNT).

According to Schmid (1975), the following procedure creates micronuclei in the cytoplasm. As the central components travel toward the spindle poles during anaphase, acentric chromatid and chromosomal fragments trail behind. Because they lack a centromere, chromosomal fragments or acentric chromosomes that are not integrated into daughter nuclei during mitosis give rise to micronuclei. The daughter cells' nuclei may have the lagging element, but a percentage of them may be micronuclei, which are subsidiary nuclei that are significantly smaller than the main nucleus (1/5 to 1/20).

2. MATERIAL AND METHOD

2.1 Chemicals and Sample Gathering

Qualigens (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) provided the anhydrous D-glucose and zinc acetate dihydrate. Fresh mulberry leaves were gathered at Isabella Thoburn College Campus, which is situated at 26°52′18′N 80°56′32′E, Lucknow, Uttar Pradesh, India. We used Bisphenol A with a purity of > 98% that was produced by SIGMA-ALDRICH, Co., 3050 Spruce Street, St. Louis, MO 63103, USA (314-771-5765). BPA was added to ethanol to create a test solution that would offer a specific stock solution for each concentration.

2.2 Preparation of *Morus nigra* Aqueous Leaf Extract

The leaves were cleaned, let to air dry, and then ground into a powder. 200 mL of Milli-Q water and 15 g of leaf powder were heated at 60°C for around 30 minutes. The extract was filtered and kept at 4°C following cooling and an overnight incubation period.

2.3 Glucose-Capped Zinc Oxide Nanoparticles (G-ZNPs): Biogenic Production

According to published research, *Morus nigra* leaf extract has been used to create ZnO nanoparticles. 100 milliliters of Milli-Q water were used in this investigation to dissolve 0.5 M zinc acetate dihydrate. At 70°C, 25 mL of leaf extract was added dropwise while being stirred magnetically. An hour later, 2.2 g of glucose was added gradually, and the mixture was agitated for two to three hours. After centrifugation, the mixture was cleaned, dried at 70°C, calcined at 400°C, and then ground.

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2.4 TEST ANIMAL

The test subject was the snakehead, *Channa punctatus*, a predatory fish belonging to the Channidae family. This fish was selected for the experiment because it is readily available (it can be used for experiments all year round), breathes air, has a high sensitivity to toxic substances, shares 85% of human physiology, can tolerate stressful situations, is reasonably priced, and is mostly consumed by economically disadvantaged populations. It is of least concern, according to the IUCN Red List of Threatened Species database (IUCN 2014).

2.5 COLLECTION OF TEST ANIMAL AND ACCLIMATIZATION OF FISH

Fish was acclimatized to the lab environment for ninety-six hours in order to acclimate. To get rid of any bacterial or fungal infections, they were treated with 4% $KMnO_4$. Before the acclimatization process, feeding was done every 12 hours. The healthy Channa punctatus fish, weighing 35± 5 g at birth and measuring 15 ± 2 cm in length, were collected from the local market in Lucknow, Uttar Pradesh, and randomly assigned to two tanks, each with 60 liters of water.

2.6 WATER LOADING RATES

Every study series kept the water volume at 4 mg/L fish weight.

2.7 EXPERIMENTAL SETUP

Six groups are used in the experiment to assess the effects of bisphenol A and *M. nigra* extract separately and together. Group 1 is the control group that receives no therapy. Group 2 is given only M. nigra extract at a dosage of 3.0 mg/l in order to assess its independent effect. Group 3 is solely exposed to 0.72 mg/l of bisphenol A in order to monitor its effects. Groups 4, 5, and 6 are treated with a combination of Bisphenol A (0.72 mg/l) and three different concentrations of *M. nigra* extract (1.0 mg/l, 2.0 mg/l, and 3.0 mg/l, respectively) to see if different extract dosages can mitigate or offset the effects of Bisphenol A. A comparison of the control, individual treatments, and their combinations at various extract levels is made possible by this configuration.

2.8 MN Slide Preparation

0.05–0.1 ml of blood was extracted by cardiac puncture using a heparinized syringe (1 ml). Using another glass slide, created a thin smear of blood on a previously cleaned slide. The

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slides were air-dried overnight at ambient temperature in a dry, dust-free environment. The slides were fixed by immersing them in pure methanol for five to ten minutes. The slides were air-dried for a minimum of one hour. Stained the slides for two to three minutes using May-Grunnwald's Solution 1. Slides were cleaned with DD water and dried. Stained the slides for three to six minutes using May-Grunnwald's Solution 2. D.D. water was used to wash and dry the slides. The slides were stained for 30 minutes with 6–10% Giemsa stain in phosphate buffer (working solution). The slides were meticulously cleaned with DDW to get rid of all the Giemsa particles. The slides were air dried overnight. DPX-mountant was used to make the slides permanent, and they were dried overnight at 60 ºC on a hot plate. Using 40/10 X objective lenses, the slides were examined under a microscope, and the micronucleated cells were scored.

2.9 STATISTICAL ANALYSIS

A statistical analysis program (SPSS 26.0 for Windows) was used. Every data point was displayed as mean ± SE. The significance of the difference between the control and treatment groups was evaluated using a one-way ANOVA and posthoc Tukey's test. Statistical significance was defined as P values of less than 0.05.

3. RESULT

LC_{50} determination of BPA

After 96 hours of exposure, the percentage of fish that died at various BPA doses was calculated. The proper concentration range was chosen during a range-finding test. Additionally, the experimental fish were divided into batches of ten and exposed to distinct BPA doses ranging from 4 mg/l to 12 mg/l. Fish mortality was noted at 24, 48, 72, and 96 hours after BPA exposure. To keep the dissolved oxygen concentration at the ideal level throughout the experiment, the test chemical was replaced every day. To confirm the findings, the experiment was conducted three times in duplicates. The computer program Probit Analysis was used to determine the median fatal concentration (Finney, 1971). The LC_{50} value was computed after the percentage mortality of *C. punctatus* was found to be 0% and 100% at BPA concentrations of 4 mg/l and 12 mg/l, respectively.

Induction of MN during Experiment

Fish in Group 1, the control group, had estimated micronucleus frequencies in their blood erythrocytes of 0.13 ±0.16, 0.16 ±0.01, 0.14 ±0.02, and 0.16 ±0.01 after 24, 48, 72, and 96

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hours. When compared to Group 1, the micronucleus frequencies in blood erythrocytes in Group 2 treated with *Morus nigra* (3 mg/l) were calculated to be 0.14 ±0.01, 0.13 ±0.01, 0.15 ±0.01, and 0.13 ±0.01 after exposure periods of 24, 48, 72, and 96 hours, respectively. After all exposure periods, Group 3 treated with a sublethal dosage of Bisphenol A (0.72 mg/l) showed significantly ($p < 0.05$) higher micronuclei frequencies than Group 1. After 96 hours of exposure, Group 3 had the highest frequency of micronuclei (0.94±0.02). Additionally, all subsequent exposure periods between 24, 48, 72, and 96 hours showed significant variations ($p<0.05$). In contrast to the MN frequency caused by Bisphenol A alone in Group 3 during all subsequent exposure periods, fish exposed to sublethal concentrations of Bisphenol A with extract from *Morus nigra* leaves significantly ($p >0.05$) decreased by all three tested concentrations (1, 2, and 3 mg/l) of *Morus nigra* in Groups 4, 5, and 6.

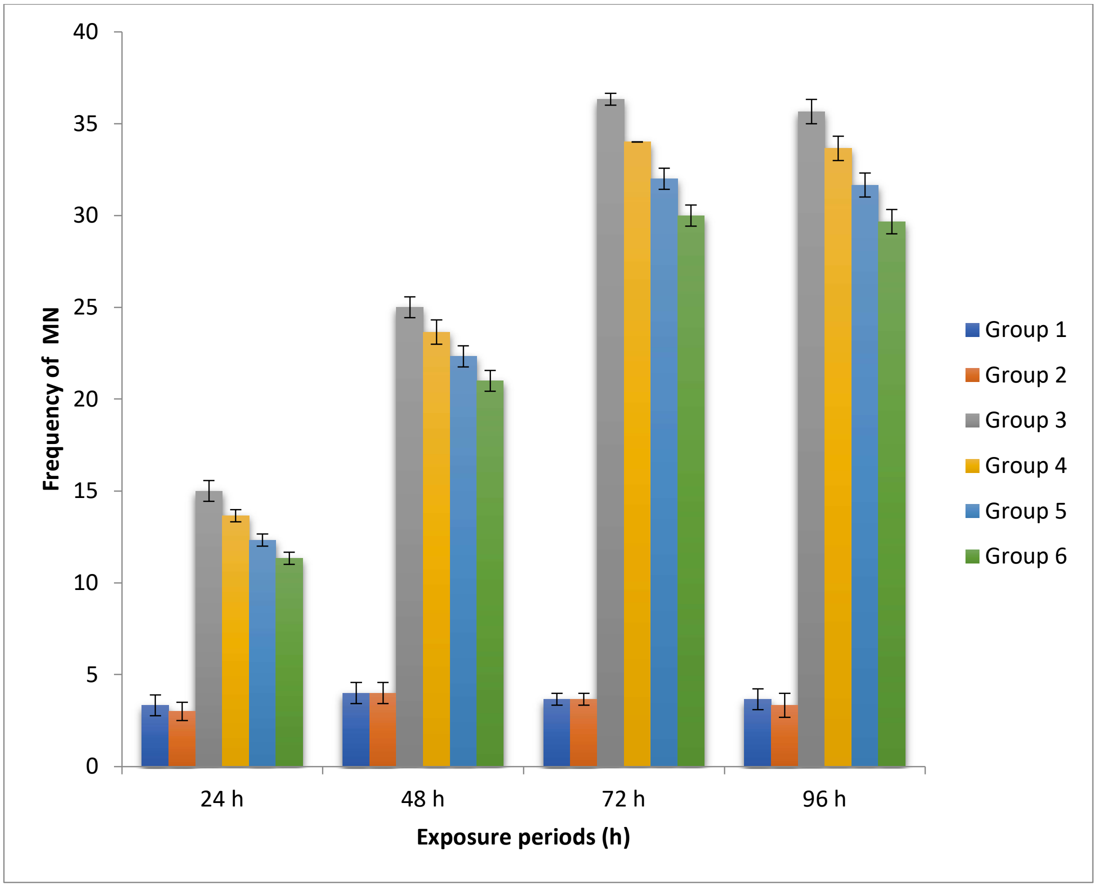

Fig: The frequency of MN induced by Bisphenol A and reduced by *Morus nigra* extract in erythrocytes of *C. punctatus*

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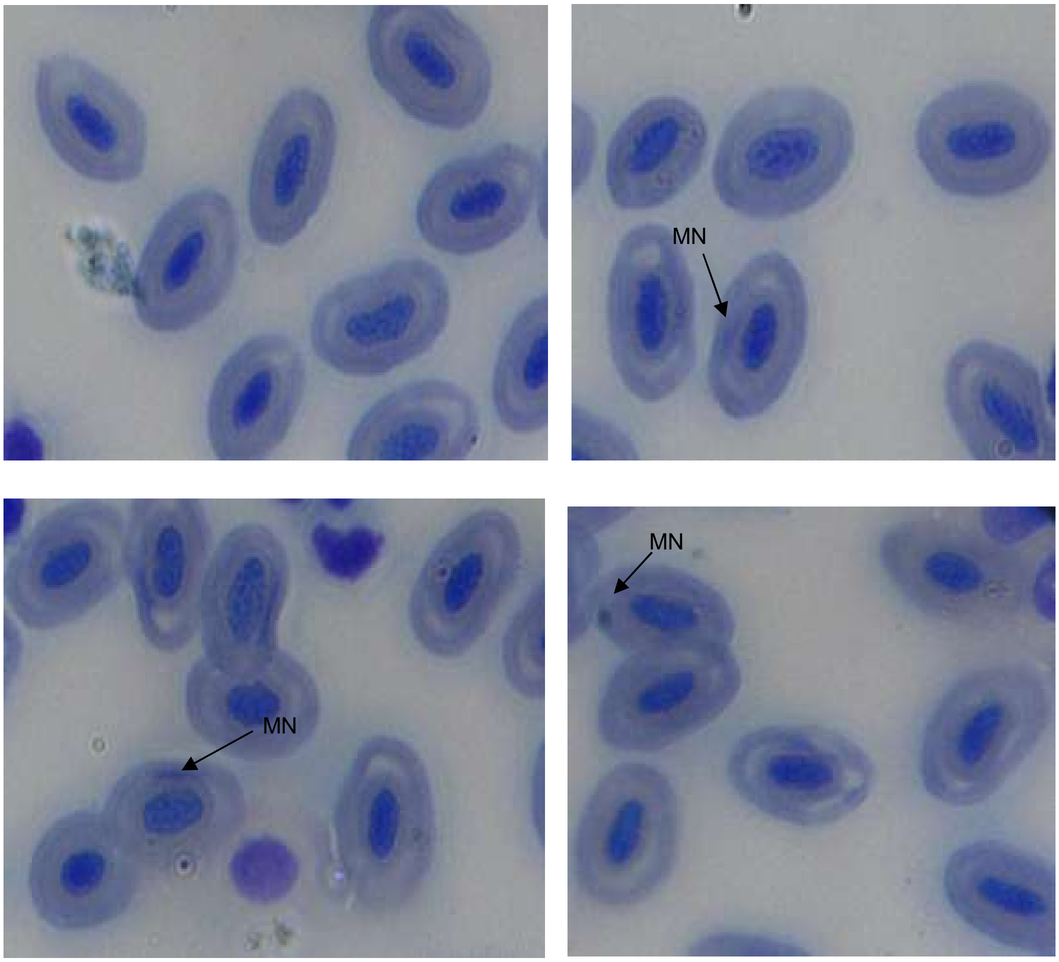

Fig: Showing micronuclei in erythrocytes of *C. punctatus* induced by Bisphenol A

4. DISCUSSION

The current work shows that exposure to the well-known endocrine disruptor bisphenol A (BPA) causes major genotoxic effects in *Channa punctatus*, as shown by an increased frequency of micronuclei in erythrocytes. A commonly used biomarker for identifying chromosomal damage, breakage, or mitotic spindle failure, the micronucleus assay's rise unmistakably shows toxicant-induced DNA damage.

Fish exposed to BPA (0.72 mg/L; LC_{50}/10) in the current study exhibited a substantial increase ($p < 0.05$) in the frequency of micronuclei at all exposure durations (24–96 h), with the maximum induction recorded after 96 hours (0.94 ± 0.02). This demonstrates that BPA has the potential to be genotoxic to aquatic life. Reactive oxygen species (ROS) are produced as a result of oxidative stress, which is the main mechanism behind BPA-induced genotoxicity. These ROS can result in chromosomal abnormalities, breakage in DNA strands, and eventually the creation of micronuclei. Fish treated with glucose-capped ZnO nanoparticles mediated by *Morus nigra* aqueous leaf extract, on the other hand, did not exhibit any discernible difference in micronuclei frequency as compared to the control group. This implies that the produced nanoparticles are physiologically safe in the current experimental setup and non-genotoxic at the tested concentration (3 mg/L). Crucially, compared to BPA-treated groups, co-treatment of BPA with various glucose-capped ZnO nanoparticles concentrations (1, 2, and 3 mg/L) significantly decreased the frequency of micronuclei. The protective effect was shown to be dose-dependent, with a concentration of 3 mg/L showing the largest reduction. This demonstrates the antigenotoxic potential of glucose-capped ZnO nanoparticles produced from *Morus nigra*. There are several reasons for glucose-capped ZnO nanoparticles protective mechanism. Bioactive phytochemicals like flavonoids, phenolics, and antioxidants, which are abundant in *Morus nigra* leaves, are essential for scavenging free radicals and lowering oxidative stress. These phytoconstituents serve as stabilizing and capping agents during the manufacture of nanoparticles, increasing the biological activity of ZnO nanoparticles. The antioxidant and protective qualities of zinc oxide, such as its capacity to maintain cellular membranes and shield DNA from oxidative damage, are well-known. Fish's antioxidant defense mechanism is probably strengthened by the combination of ZnO nanoparticles and plant-derived bioactive chemicals, which lowers micronuclei generation and ROS-mediated DNA damage. The observed dose-dependent reduction in genotoxicity can be explained by this synergistic interaction. Additionally, the results of this study are in line with earlier findings that show plant-mediated nanoparticles have strong protective effects against environmental pollutants. Utilizing green-synthesized nanoparticles provides an efficient and sustainable method of reducing pollution-induced toxicity in aquatic environments.

5. CONCLUSION

The current work demonstrates that Bisphenol A (BPA) significantly increases the frequency of micronuclei in erythrocytes across all exposure durations, indicating that BPA causes genotoxic effects in the freshwater fish *Channa punctatus*. This suggests that exposure to BPA causes genomic instability and chromosomal damage in aquatic creatures.

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The biocompatibility of biogenically produced glucose-capped ZnO nanoparticles obtained from *Morus nigra* aqueous leaf extract was demonstrated by their non-genotoxic nature at the measured concentration. Interestingly, co-treatment with glucose-capped ZnO nanoparticles dramatically decreased the development of BPA-induced micronuclei in a dose-dependent manner; the highest level of protection was seen at 3 mg/L. These results imply that glucose-capped ZnO nanoparticles mediated by *Morus nigra* have substantial antigenotoxic potential and can successfully reduce DNA damage caused by BPA, most likely by strengthening cellular defense systems. Overall, the study emphasizes how green-synthesized ZnO nanoparticles may be exploited as environmentally beneficial and efficient agents to lessen genotoxic stress brought on by environmental contaminants. This study lays the groundwork for future investigations into the uses of green nanotechnology in aquatic toxicity and bioremediation, as well as the growing significance of this technology in environmental protection.

6. CONFLICTING INTERESTS

The authors assert that there are no competing interests.

7. CONTRIBUTION OF AUTHORS

Vivek Kumar is responsible for the experiment's design, layout, manuscript editing, data analysis utilizing statistics and biochemical index analysis, data interpretation, reference management, methodology, validation, and supervision. Amisha Verma set up the experiment, drafted the manuscript, conducted the experiment, handled the fish specimens, collected raw data in the wet lab, and conceptualized, reviewed, and edited the manuscript. The two authors reviewed the work.

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