

Micronucleus Assay for Detecting Genotoxic Stress in Freshwater Fish (*Heteropneustes fossilis*) Under Tannery Effluent Exposure

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ABSTRACT

Industrial tannery effluents are major sources of chromium and other persistent pollutants that pose serious threats to freshwater ecosystems because they cause cytotoxicity and genotoxicity in aquatic species. The current study used the erythrocyte micronucleus (MN) assay and erythrocyte nuclear abnormalities (ENAs) as sensitive biomarkers to examine the genotoxic effects of treated tannery effluent in the freshwater catfish *Heteropneustes fossilis*. Under controlled laboratory conditions, fish were exposed to sub-lethal concentrations of tannery effluent (2% TE, 5% TE, 10% TE, and 15% TE) for 15, 30, and 45 days. The May–Grünwald–Giemsa staining method was used to prepare peripheral blood smears, which were then microscopically analyzed for cytological and nuclear changes. Control fish displayed normal erythrocytes with intact nuclei, while exposed fish displayed a concentration-dependent rise in cellular and nuclear abnormalities. Fish exposed to 2% effluent showed modest cytotoxic modifications, such as nuclear and cell morphological abnormalities and cellular elongation. Exposure to 5% effluent produced unique nuclear lesions, while exposure to 10% effluent caused notable micronucleus production, nuclear abnormalities, and morphological disturbance of erythrocytes. Extensive chromosomal instability and DNA damage were indicated by the severe genotoxic effects of the highest concentration (15%), which included numerous micronuclei, blebbed nuclei, nuclear deformities, and noticeable erythrocyte abnormalities. These changes are attributed to oxidative stress brought on by chromium and related hazardous pollutants found in tannery wastewater. The MN assay is a quick, accurate, and affordable biomarker for evaluating environmental genotoxicity, as evidenced by the steady rise in micronuclei and nuclear abnormalities with increasing effluent concentration. The results highlight the ecological concerns connected to tannery effluent discharge and establish *Heteropneustes fossilis* as a suitable sentinel species for freshwater biomonitoring, underscoring the necessity of efficient wastewater treatment and ongoing environmental monitoring.

Keywords: *Heteropneustes fossilis*, tannery effluent, micronucleus assay, genotoxicity, chromium, oxidative stress

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INTRODUCTION

Industrial growth drives development but also releases treated and untreated wastes that can harm ecosystems, especially aquatic environments. Human activities introduce pollutants such as heavy metals (As, Hg, Cd, Cr, Pb) [1]. Among the most prevalent aquatic pollutants are toxic metals, which are mutagenic and carcinogenic. They remain in the environment for a very long period because they are persistent and non-biodegradable [2]. According to a number of studies, toxic metal contamination has recently been linked to changes in fish and other aquatic fauna morphology, biochemistry, physiology, and behaviour [3-6]. Fish are frequently employed to trace the impacts of heavy metals in the area and are at the top of the aquatic food chain.

Micronucleus analysis is a simple and effective method for assessing cytogenetic damage in indicator animals used for ecological monitoring [7,8]. Animals with nucleated erythrocytes, such as fish, are valuable bioindicators because they exhibit micronuclei and other nuclear abnormalities [9]. Environmental pollution increases mutation rates and genetic damage in animal populations. Bio-testing and bioindication methods help rapidly evaluate the biological effects of anthropogenic stressors on ecosystems. Ecotoxicological studies provide insights into toxicant-induced responses at the genetic, organismal, and population levels [10,11].

Fish are perfect model animals for genotoxicological study because they are early indicators deterioration caused by toxicants [12]. Along with the bioconcentrating capability

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of chromium, several authors have reported on the cytotoxicity and genotoxicity of effluents containing chromium in diverse investigations. According to in vitro cytological research numerous cytological indices, including cell mortality, viability, morphology, metabolism, attachment or detachment, proliferation, are impacted by metal toxicity. The alterations may directly impact fish blood parameters. According to reports, the development of micronucleus (MN) in the erythrocyte is thought to be a sign of pollution stress [13]. A tiny chromatin body in the cytoplasm called the micronucleus (MN) is created outside the central nucleus during nuclear division and is made up of condensed acentric chromosomal fragments. Additionally, cellular and nuclear abnormalities-differences from the normal structure of cells and the nucleus of erythrocytes-are used to evaluate the stress brought on by external contaminants [14]. Additional nuclear anomalies, such as notched, blebbed, lobed, and binucleated nuclei, have been noted as possible markers of cytotoxicity [15,16]. According to [17], Nile tilapia exposed to Zn, Pb, Cu, and Cd showed an increase in the frequency of micronuclei.

This genotoxicity is assessed using biomarkers such as micronucleus (MN), erythrocyte nuclear abnormalities (ENA), and Comet assays [18,19]. Monitoring these biological responses helps evaluate pollutant impact and overall aquatic ecosystem health [20,21]. Cytogenetic homeostasis in indicator animals is crucial for ecological monitoring, with micronucleus analysis being a simple and effective method [22]. Animals with nucleated erythrocytes are commonly used, and along with micronuclei, various nuclear and cellular abnormalities are assessed. Environmental pollution increases mutation rates and genetic load in populations, making bioindicator species valuable natural tools for monitoring environmental health [23]. Bio-testing and bioindication are effective approaches for rapidly assessing complex biological responses of ecosystems to anthropogenic stressors. Ecotoxicological studies on natural populations enable integrated evaluation of toxic effects at population, organismal, and genetic levels. Cytogenetic biomarkers, particularly in the peripheral blood of vertebrates (fish, amphibians, reptiles, and birds), are widely used to detect mutagenic impacts of environmental pollution. Among various methods for assessing genome instability, the micronucleus (MN) test is preferred due to its simplicity, reliability, and applicability under field conditions [24]. It evaluates cytogenetic homeostasis by quantifying micronuclei frequency and other nuclear abnormalities in cells. The MN test is independent of species karyotype, suitable for tissues with low mitotic activity, and allows non-invasive, in vivo monitoring over time. Its sensitivity makes it a universal indicator of environmental contamination and clastogenic effects of chemical and physical agents. Recent advancements include micronucleus cytoanalysis, which provides a more comprehensive assessment of nuclear damage and genome instability, commonly applied in binucleated lymphocytes and epithelial cells. Overall, these methods significantly

enhance environmental monitoring and risk assessment in polluted ecosystems

MATERIAL & METHODS

Sample Collection and Experimental Design: In a Tarson plastic container, the treated tannery effluent had been collected from Banthar, Unnao District, Uttar Pradesh, India. Fishermen from surrounding water bodies in Kanpur will assist in collecting the experimental fish, *Heteropneustes fossilis*. For 15 days, 30 days, and 45 days, fish will be introduced into aquariums with only water (group I, control) and four sub-lethal concentrations (group II 2%, group III 5%, group IV 10%, and group V 15% Tannery Effluents).

Micronucleus Test: Small, extranuclear structures known as micronuclei are created during mitosis from chromosomes or acentric chromosomal fragments that are absent from both daughter nuclei. Micronuclei test (MNT) by Giemsa stain [25].

Collection of Blood from Fish: Rinsed the syringe with 75 U/ml of heparin solution, leaving a gap of about 2 mm at the top of the syringe column. The needle was pricked in the centre of the caudal peduncle's ventral side, reaching up to the vertebral column. As soon as the blood began to flow into the syringe, the dispenser was gently pulled outward to collect the blood.

Preparation of Micronuclei Slides: Take a heparinised syringe (1 ml) and extract 0.05–0.1 ml of blood via heart puncture or caudal vein. Make a thin smear on the precleaned slide with the help of a blood smear slide. The air-dried slides were kept at room temperature overnight in a dust- and moisture-free environment. The slides were fixed by immersing them for five to ten minutes in methanol. Air-dried the slides for at least 1 hour. Stained the slides in May-Grunwald's Solution 1 for 2 to 3 min. Washed the slides with DD Water and dried. Stained the slides in May-Grunwald's Solution 2 for 3 to 6 min. Washed the slides with DD Water and dried. Giemsa stain (6–10%) was applied to the slides for 30 minutes in phosphate buffer (working solution). Carefully cleaned the slides with DDW to get rid of all the Giemsa particles. Overnight, let the slides air dry. DPX-mount was used to make the slides permanent, and they were then dried overnight at 60 °C on a hot plate. The micronucleated cells were scored after the slides were examined using a microscope with 40/10 X objective lenses.

Scoring of MN Slides: Only MN that were clearly separate from the main nucleus, had obvious borders, and had the same colour as the nucleus—not more than one-third of the diameter of the main nucleus were scored.

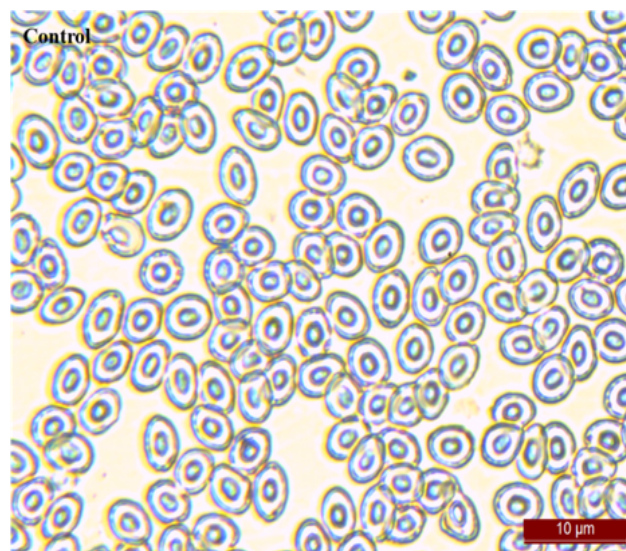
RESULTS

The erythrocytes of *Heteropneustes fossilis* exposed to 2% tannery effluent for 45 days showed mild cytological alterations compared to the control group. Treated fish exhibited changes in cell and nuclear size along with cellular elongation (CE), whereas control fish showed

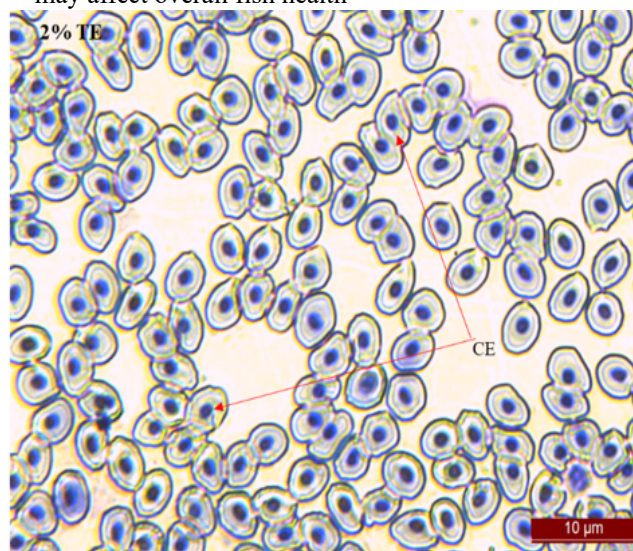
normal erythrocytes and intact nuclei. These findings indicate early cytotoxic effects caused by tannery effluent and suggest that even low concentrations can induce cellular stress in fish blood cells.

The erythrocytes of *Heteropneustes fossilis* exposed to 5% tannery effluent for 45 days showed noticeable nuclear lesions (NL) and alterations in cell and nuclear

morphology compared to the control group, which exhibited normal erythrocytes and intact nuclei. These abnormalities indicate early genotoxic and cytotoxic effects caused by toxic pollutants, particularly chromium and heavy metals present in the tannery effluent. The findings suggest that even low concentrations of tannery effluent can induce cellular damage in fish blood cells and may affect overall fish health

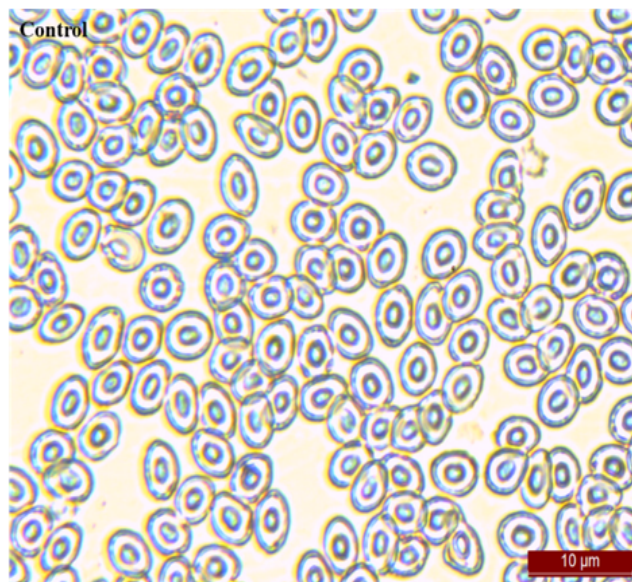


Control treatment on string catfish, *Heteropneustes fossilis* show normal cells and nucleus

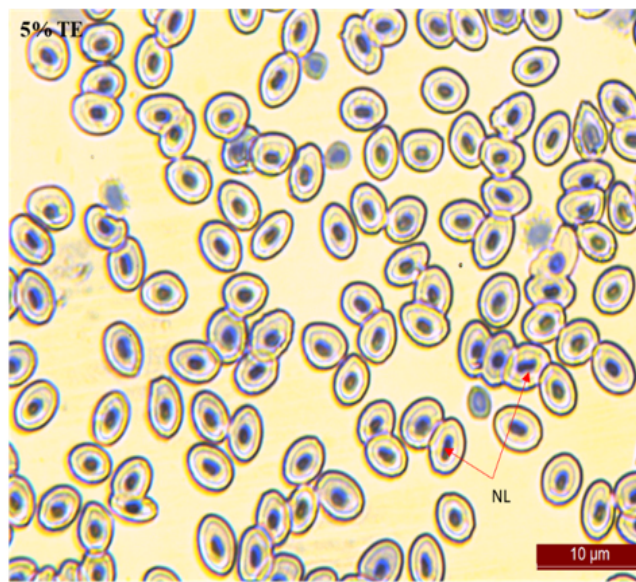


2%TE treatment on string catfish, *Heteropneustes fossilis* show change size of cells and nucleus

Fig.1: Microscopic images of Giemsa-stained blood smears from control and exposed to (2% TE) tannery effluent on string catfish, *Heteropneustes fossilis*

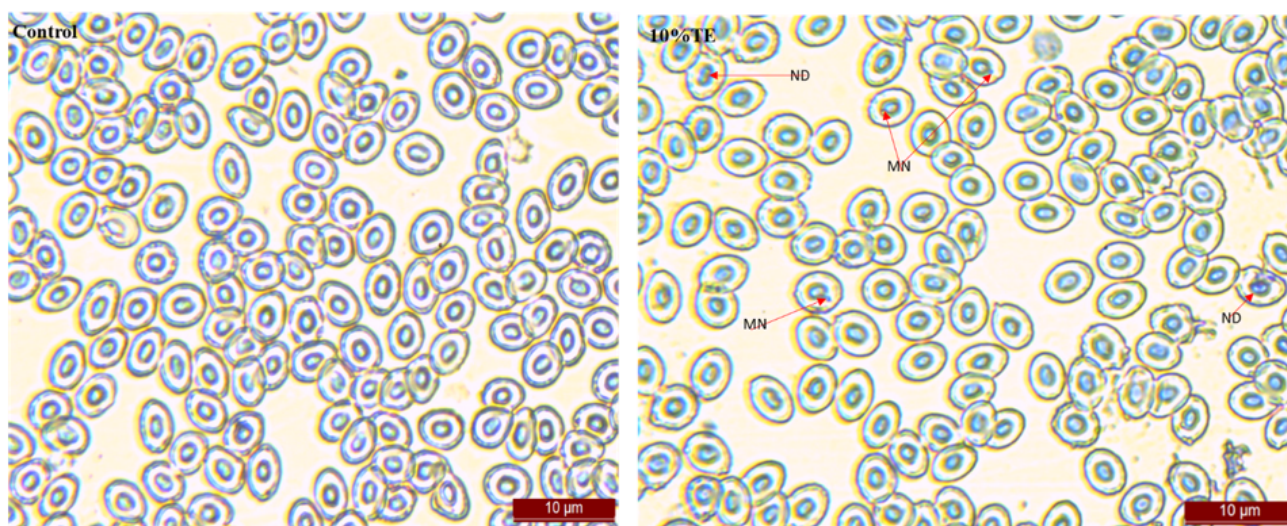


Control treatment on string catfish, *Heteropneustes fossilis* show normal cells and nucleus



5%TE treatment on string catfish, *Heteropneustes fossilis* show change size of cells and nucleus

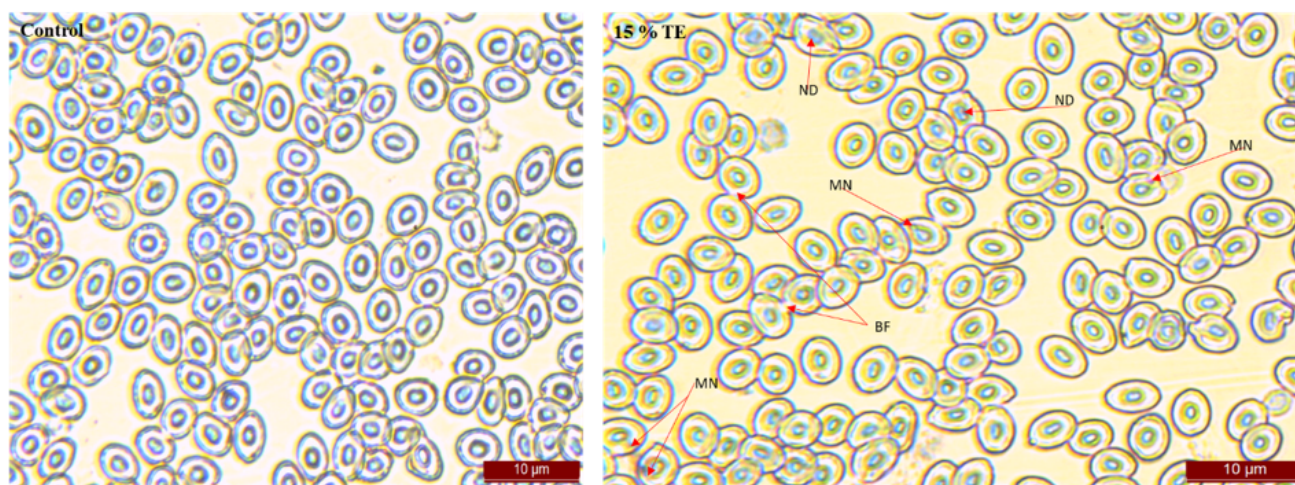
Fig.2: Microscopic images of Giemsa-stained blood smears from control and exposed to (5% TE) tannery effluent on string catfish, *Heteropneustes fossilis*



Control treatment on string catfish, *Heteropneustes fossilis* show normal cells and nucleus

10%TE treatment on string catfish, *Heteropneustes fossilis* show change size of cells and nucleus

Fig.3: Microscopic images of Giemsa-stained blood smears from control and exposed to (10 % TE) tannery effluent on string catfish, *Heteropneustes fossilis*



Control treatment on string catfish, *Heteropneustes fossilis* show normal cells and nucleus

15%TE treatment on string catfish, *Heteropneustes fossilis* show change size of cells and nucleus

Fig.4: Microscopic images of Giemsa-stained blood smears from control and exposed to (15% TE) tannery effluent on string catfish, *Heteropneustes fossilis*

The micronucleus assay in erythrocytes of *Heteropneustes fossilis* exposed to 10% tannery effluent for 45 days showed significant cytological abnormalities compared to the control group. Control fish exhibited normal erythrocytes with intact nuclei, whereas treated fish showed increased micronuclei (MN), nuclear deformities (ND), and alterations in cell and nuclear size. These abnormalities indicate genotoxic and cytotoxic effects from prolonged exposure to tannery effluent, particularly from chromium and associated heavy metals. The findings confirm that micronucleus formation can serve as an effective biomarker for assessing aquatic pollution and heavy metal toxicity.

The erythrocytes of *Heteropneustes fossilis* exposed to 15% tannery effluent for 45 days showed severe genotoxic alterations compared to the control group. Treated fish exhibited increased micronuclei (MN), nuclear deformities (ND), blebbed nuclei (BN/BF), and changes in cell and nuclear morphology, whereas control fish showed normal erythrocytes with intact nuclei. These abnormalities indicate significant DNA damage and cytotoxic effects caused by chromium and other toxic pollutants present in tannery effluent. The findings confirm the strong genotoxic potential of tannery effluent and suggest that the micronucleus assay is an effective biomarker for monitoring aquatic pollution and heavy metal toxicity.

DISCUSSION

The current study showed a concentration-dependent rise in erythrocytic abnormalities in *Heteropneustes fossilis* following 45 days of exposure to tannery effluent, indicating progressive cytotoxic and genotoxic damage. Fish treated with 2% tannery effluent displayed moderate modifications such as cellular elongation and changes in cell and nuclear size, whereas 5%, 10%, and 15% tannery effluent showed noticeable nuclear lesions, micronuclei, nuclear deformities, and blebbed nuclei. These results unequivocally show that even at relatively low concentrations, extended exposure to tannery effluent can impair erythrocyte integrity and cause DNA damage.

Fish exposed to 2% tannery effluent exhibited moderate cytological changes that may indicate the onset of cellular stress responses before the emergence of severe nuclear damage. Erythrocyte morphological abnormalities, such as variations in cell size, nuclear size, and cellular elongation, are considered to be sensitive markers of oxidative stress and membrane instability carried on by environmental pollutants. Higher levels of chromium, salts, sulphides, phenols, and other heavy metals are known to be present in tannery effluents. These metals produce reactive oxygen species (ROS), which cause lipid peroxidation and cellular architecture disturbance. The current findings are corroborated by reports of oxidative stress-induced erythrocyte membrane damage as an early reaction to heavy metal exposure in a number of fish species [26, 27].

Nuclear lesions and noticeable changes in nuclear morphology at 5% tannery effluent signify the beginning of genotoxic consequences. Chromosome instability and hindered nuclear division are regarded as intermediary stages of nuclear abnormalities, such as lobed, notched, or malformed nuclei. Heavy metals, especially chromium, have the ability to directly interact with DNA, causing oxidative lesions, strand breakage, and inhibition of DNA repair enzymes, all of which can lead to nuclear abnormalities. Hexavalent chromium [Cr (VI)] is well known for its potent mutagenic and carcinogenic qualities because it may pass through cell membranes and create extremely reactive intermediates during intracellular reduction. Similar increases in erythrocytic nuclear abnormalities after exposure to chromium have been documented in freshwater fish and are commonly recognized as markers of genotoxicity caused by pollutants. [28,29].

The genotoxic potential of tannery wastewater is further supported by the notable rise in micronuclei and nuclear abnormalities in fish exposed to 10% tannery effluent. Micronuclei are a trustworthy marker of chromosomal damage because they are produced when chromosome fragments or entire chromosomes fail to integrate into daughter nuclei during mitosis. The micronucleus assay is one of the most used biomarkers for tracking aquatic genotoxicity because fish erythrocytes are nucleated and circulate for long periods of time. Fish exposed to industrial effluents, heavy metals, pesticides, and urban wastewater have been shown to have elevated

micronucleus frequencies, indicating the assay's sensitivity in environmental biomonitoring. Thus, the current results are in line with earlier research that found micronucleus induction as a result of oxidative stress and chromium-mediated DNA damage [30,31].

Fish exposed to 15% tannery effluent showed significant cytotoxicity and substantial DNA damage, as evidenced by the severe prevalence of micronuclei, nuclear abnormalities, and blebbed nuclei. Blebbed nuclei are often associated with irreversible nuclear membrane damage, apoptosis, and advanced stages of cellular degeneration. Their presence at the highest effluent concentration indicates that the enormous oxidative burden caused by tannery pollutants was too much for *H. fossilis*'s antioxidant defense mechanisms to handle. The biochemical changes seen in the current study, such as elevated oxidative stress indicators and modifications in antioxidant enzyme activity, give more credence to this hypothesis. The significant erythrocytic abnormalities observed in the high-exposure groups may be due to persistent oxidative stress, which can also lead to chromosomal aberrations, DNA fragmentation, and apoptotic pathways [32, 33].

Strong cytotoxic and genotoxic characteristics of tannery effluent are confirmed by the concentration-dependent rise in erythrocytic abnormalities found in this study. In fish erythrocytes, oxidative stress, chromosomal damage, and nuclear instability seem to be largely caused by chromium and related heavy metals. For evaluating water pollution and tracking the ecological concerns connected to tannery effluent discharge, the micronucleus and erythrocytic nuclear abnormality assays can be regarded as extremely sensitive and economical biomarkers. The results also highlight the possible risk that untreated or insufficiently treated tannery wastewater poses to freshwater habitats and aquatic life.

CONCLUSION

The present study revealed that tannery effluent causes concentration-dependent cytotoxic and genotoxic effects in *Heteropneustes fossilis*. Exposure to different concentrations of tannery effluent induced various erythrocyte abnormalities, including cellular elongation (CE), nuclear lesions (NL), micronuclei (MN), nuclear deformities (ND), and blebbed nuclei (BN/BF).

These alterations indicate DNA damage and cellular stress mainly caused by chromium and other toxic pollutants present in tannery wastewater. The findings confirm that the micronucleus assay is an effective biomarker for monitoring aquatic pollution and heavy metal toxicity

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