ISSN: 0976-4933 (Print)
Journal of Progressive Science
A Peer-reviewed Research Journal
Vol.17, No.01, pp 50-63 (2026)
https://doi.org/10.21590/jps.17.01.04

Arsenic-Induced Antioxidant Enzyme Responses in Catfish, *Heteropneustes fossilis*

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Abstract

Arsenic contamination in groundwater is a significant environmental challenge worldwide, affecting both developed and developing nations. Aquatic organisms, including fish, are also severely affected. Arsenic accumulates in fish tissues such as gills, liver, kidneys, skin, and muscles, causing damage to these vital organs of the fish. In the present study, changes in the activity of two antioxidant enzymes, i.e., superoxide dismutase (SOD) and catalase, were measured in the liver of catfish, *Heteropneustes fossilis*. The fish were exposed to two sublethal concentrations of dibasic sodium arsenate heptahydrate: 4.88 mg L^{-1} and 14.64 mg L^{-1}. Fish were treated for 96 hour and 7 days with both concentrations. The activity of SOD and catalase was measured by the spectrophotometric method. Our data showed about a 3.5-fold increase in SOD activity in fish exposed to 4.88 mg L^{-1} arsenic for 96 hour. No significant increase was observed in fish exposed to the higher concentration (14.64 mg L^{-1}). Fish exposed to arsenic for 7 days exhibited about 2-fold and 1.4-fold SOD activity at 4.88 mg L^{-1} and 14.64 mg L^{-1}, respectively. The catalase assay showed nearly 2-fold increased activity in fish exposed to arsenic solutions of 4.88 mg L^{-1} and 14.64 mg L^{-1} for 96 hour. However, after 7 days of treatment, fish exhibited only a mild increase in catalase activity ($\sim$ 1.25-fold) compared to the control group. The findings of the study suggest that SOD and catalase enzyme activity vary with arsenic concentration and treatment duration in *H. fossilis*.

Keywords and Phrases: Arsenic, Heteropneustes, SOD assay, catalase assay.

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Introduction

Arsenic contamination represents one of the most widespread and complex environmental challenges affecting both developed and developing countries. Arsenic, a naturally occurring metalloid, is present in over 200 minerals, primarily as sulfides and oxides. Its pervasive distribution in groundwater, surface water, soil, sediments, and biota, combined with its established carcinogenic and multi-system toxic properties, has elevated arsenic as a contaminant of global concern Shaji et al. (2021). Aquatic organisms may be exposed to arsenic through direct absorption from water, ingestion of contaminated sediments, or via trophic transfer within the food web. Fish play a vital role in aquatic ecosystems due to their ecological functions, relatively long lifespans, and significance as a food source for both humans and other animals. Arsenic can accumulate in various fish tissues, including gills, liver, kidneys, skin, and muscles. Tissue-specific accumulation patterns reflect differences in uptake mechanisms, binding affinities, and detoxification capacities Authman et al. (2015). The accumulation of arsenic in edible fish tissues raises significant concerns regarding food safety and human exposure. Aquatic organisms have long served as indicators of environmental quality, with fish occupying a central role in ecotoxicological studies. Their ecological importance, sensitivity to pollutants, ability to bioaccumulate contaminants, and nutritional value make fish effective bioindicators of aquatic pollution. In areas affected by arsenic contamination, fish-based assessments provide an essential connection between environmental exposure and biological effects, facilitating evaluation of ecosystem health and potential risks to human populations.

Fish residing in arsenic-contaminated aquatic environments are exposed to arsenic through direct contact with water, ingestion of contaminated sediments, and dietary intake. Benthic and bottom-dwelling species are especially susceptible because of their continuous interaction with sediments, which frequently act as arsenic reservoirs. Prolonged exposure results in arsenic accumulation within fish tissues, causing sub-lethal toxic effects that impair growth, reproduction, and survival. Although these effects may not be immediately observable, they can produce lasting impacts on fish populations and overall fisheries productivity Kumari et al. (2017).

The liver and skin are critical target organs for evaluating arsenic toxicity in fish. The liver functions as the primary site for metabolism, detoxification, and storage of xenobiotics, including arsenic. Consequently, hepatic tissues are highly sensitive to toxic insults, and histopathological alterations in the liver offer clear evidence of chronic exposure and physiological stress. In contrast, the skin constitutes the initial interface between fish and their environment. As a multifunctional organ responsible

for protection, osmoregulation, respiration, and immune defense, fish skin is particularly susceptible to waterborne contaminants. Pathological changes in skin tissues frequently reflect early responses to environmental stress and can serve as sensitive indicators of aquatic pollution. Physiologically, fish possess detoxification systems similar to those of higher vertebrates, including enzymatic pathways for biotransformation, antioxidant defense, and metal sequestration. Arsenic exposure disrupts these systems by impairing enzyme activity, inducing oxidative stress, and altering cellular homeostasis Flora (2011). These biochemical disturbances often precede observable pathological changes, establishing fish as a sensitive early-warning system for environmental contamination. When integrated with histopathological analysis, biochemical biomarkers in fish provide robust evidence of pollutant-induced stress and tissue damage Kumari et al. (2017).

Heteropneustes fossilis, commonly referred to as the stinging catfish or "Singhi," is a native freshwater species widely distributed throughout India, Bangladesh, Nepal, and other regions of South and Southeast Asia. Numerous studies have documented the accumulation of heavy metals such as arsenic, cadmium, lead, and mercury in *H. fossilis*, demonstrating its capacity to bioaccumulate contaminants from polluted environments Javed & Usmani (2016). Despite these findings, the majority of research has concentrated on quantifying metal concentrations in tissues or assessing general physiological responses, while comparatively few studies have provided detailed histopathological analyses of organ-specific damage, especially in field-collected specimens from arsenic-affected areas.

Materials and Methods

Fish Collection and Acclimatization

Adult *H. fossilis* in good health and without visible injuries were used. The average length and weight (mean $\pm$ SD) were 19 ± 1.5 cm and 23 ± 1.5 g, respectively. To minimize handling stress, fish were transported in oxygenated containers from local markets. Afterwards, they were acclimated for ten to fourteen days in large glass aquariums with dechlorinated tap water, under natural photoperiod and room temperature conditions. They were fed commercial feed daily. Feces and leftover food were routinely removed, and aquarium water was changed daily to prevent waste buildup. Feeding was stopped 24 hour before the experiment to avoid feeding-related effects on arsenic exposure.

Determination of LC_{50} (96-hour)

Analytical-grade dibasic sodium arsenate heptahydrate ($Na_2HAsO_4{\cdot}7H_2O$, M.W. 312.02 g) served as the arsenic source. A stock solution of 50 mg/ml was prepared using distilled water. Fresh working concentrations were prepared from the stock solution prior to each exposure to ensure accuracy and consistency. The median lethal concentration (LC_{50}) of dibasic sodium arsenate heptahydrate for *H. fossilis* was determined over a 96-hour exposure period using standard acute toxicity testing protocols. Fish were randomly assigned to seven groups, each containing ten individuals (N= 10). The groups were exposed to the following concentrations of dibasic sodium arsenate heptahydrate: Group 1 (control): 0 mg L^{-1}; Group 2: 10 mg L^{-1}, Group 3: 20 mg L^{-1}, Group 4: 40 mg L^{-1}, Group 5: 60 mg L^{-1}, Group 6: 80 mg L^{-1}, Group 7: 100 mg L^{-1}.

Each group was maintained in a separate glass aquarium containing the designated concentration of dibasic sodium arsenate heptahydrate. Fish were exposed under static renewal conditions, with water and arsenic solution replaced every 24 hours to maintain consistent exposure levels. No food was provided during the 96-hour exposure period. Mortality was monitored and recorded at regular intervals throughout the experiment. Dead fish were promptly removed to prevent deterioration of water quality.

After 96 hour, the total number of mortalities in each group was recorded. Percentage mortality was calculated for each concentration. Mortality data were adjusted for control mortality, and the corrected percentage mortality was used for probit analysis. The LC_{50} value of dibasic sodium arsenate heptahydrate was determined using the probit method described by Miller and Tainter (1944). Adjusted percentage mortality probit values were plotted against the logarithm of arsenic concentration, and the concentration corresponding to a probit value of 5 (50% mortality) was identified from the regression line. The calculated LC_{50} value was 48.80 mg L^{-1}, corresponding to a log concentration of 1.6884.

Design of Experiments for Acute Exposure of Sub-lethal Concentrations

Two sub-lethal concentrations of dibasic sodium arsenate heptahydrate were chosen for further experiments and assessment of the toxic effect of arsenic on fish based on the calculated LC_{50} value (48.80 mg L^{-1}): Low concentration (Experimental Group 1): 10% of LC_{50} = 4.88 mg L^{-1}, High concentration (Experimental Group 2): 30% of LC_{50} = 14.64 mg L^{-1}. These doses were chosen in order to investigate the dose-dependent sub-lethal effects of arsenic without resulting in death. Two exposure

periods were chosen, i.e., 96 hrs (acute) and 7 days (short-term), to evaluate the short-term sub-chronic and acute effects of arsenic exposure. Fish were split into three groups, i.e., control, experimental 1, and experimental 2, for each exposure duration in the sub-lethal exposure study. Fishes of experimental 1 and experimental 2 were treated with 4.88 mg L^{-1} and 14.64 mg L^{-1} dibasic sodium arsenate heptahydrate concentrations, respectively. The controls remained untreated and were kept in similar conditions. Five fish of almost equal length and weight made up each group. Fish were kept in comparable laboratory settings in spotless glass aquariums. Freshly made arsenic solutions of the appropriate concentrations were administered to the experimental groups, and water was changed every 24 hours. Under the same circumstances, control fish were kept in water free of arsenic.

Assessment of Biochemical Parameters

At the end of each exposure period (96 hour and 7 days), fish were sacrificed humanely using ice-cold anaesthesia to minimize pain and stress. Immediately after sacrifice, the fish were dissected under aseptic conditions. To get rid of blood and debris, the liver and skin tissues were carefully removed and cleaned in cold physiological saline. For the biochemical assay, liver tissue from each fish was stored at -80°C. All experimental procedures were conducted following standard ethical guidelines for the use of animals in laboratory research. Efforts were made to minimize stress, suffering, and mortality of experimental animals throughout the study. Liver tissues from fish exposed to sub-lethal concentrations of arsenic under controlled laboratory conditions were selected for biochemical analysis. Liver tissue was chosen due to its central role in metabolism, detoxification, and antioxidant defense. Frozen liver samples were retrieved from storage and gently washed in 1X phosphate-buffered saline (PBS) to preserve tissue integrity. The tissues were then cleaned, dried with sterile filter paper, and accurately weighed. Pre-chilled homogenization tubes were loaded with tissue samples of known mass. Homogenization was performed using a glass-Teflon homogenizer in 1X PBS, with the process conducted on ice to prevent enzymatic degradation and preserve enzyme activity. One gram of tissue was homogenized in nine millilitres of PBS to produce a 10% (w/v) tissue homogenate. Homogenization continued until a uniform suspension was achieved. The homogenate was transferred to centrifuge tubes and centrifuged at 12,000 rpm for 20 minutes at 4°C. This step facilitated the separation of intact cells and cellular debris from the soluble protein fraction. The clear supernatant was carefully collected using a micropipette, ensuring the pellet remained undisturbed. Soluble antioxidant enzymes present in the supernatant were transferred into labelled mi-

crocentrifuge tubes. Each supernatant sample was labelled with the tissue type, exposure duration, and experimental group. Samples were stored at -80°C until further biochemical assays. Storage at ultra-low temperatures preserved enzyme activity and prevented degradation prior to analysis.

Superoxide Dismutase (SOD) Assay

SOD activity in the liver tissue of fish from control and experimental groups was measured by the spectrophotometric method using nitrite formation from superoxide radicals Das et al. (2000). A reaction mixture containing 1X PBS, L-methionine, Triton-X, Hydroxylamine hydrochloride, and EDTA was prepared, and 1.4 ml of the reaction mixture was dispensed into each test tube. Further, 100 μl of tissue sample (10% liver homogenate supernatant) was added to each test tube holding the reaction mixture. To account for background absorbance, PBS was added to control tubes in place of the sample. To allow the sample enzymes to interact with the reaction components, the reaction mixture was incubated at 37°C for five minutes. After incubation, each tube received 80 µl of riboflavin solution. Now the test tubes were exposed to light for ten minutes using an LED or CFC bulb for the photochemical process. To guarantee consistent reaction conditions for every sample, the light exposure was standardized.

Further, 1 ml of Griess reagent was added to each tube to pause the reaction and stabilize the coloured reaction product obtained. The reaction products were dispensed in triplicate in a microplate, and a plate reader (Agilent Biotek Epoch-SN) was used to measure the intensity of reaction mixtures at 543 nm. A suitable blank was used to compare readings. Depending on standards, SOD activity was expressed in units per millilitre of sample or per milligram of protein. The differences in the activity of the SOD enzyme in the controls and experimental groups were plotted as mean fold change.

Catalase Activity (CAT) Assay

The enzyme's ability to break down hydrogen peroxide (H_2O_2) was used to measure catalase activity. When heated in an acidic environment, the leftover hydrogen peroxide reacts with potassium dichromate ($K_2Cr_2O_4$) to form a chromic acetate complex detectable by spectrophotometry Sinha (1972). For the catalase assay, 500 μl of H_2O_2 was placed in dark glass test tubes. To maintain the reaction volume and pH, 625 μl of 1X PBS was added to each tube. Then, 125 μl of 10% tissue supernatant from the experimental and control groups was added and incubated at room temperature for 1 minute. During this time, the catalase enzyme in the sample

broke down hydrogen peroxide. The ingredients were gently mixed to ensure even enzyme distribution.

Next, 2 ml of potassium dichromate solution was added to each test tube to stop the reaction after incubation. Cotton plugs sealed the tubes to prevent evaporation. The tubes were incubated for ten minutes at boiling temperature (95-100°C) in a water bath. Heating facilitated the formation of the chromic acetate complex, producing a green solution proportional to the remaining hydrogen peroxide. Then, 300 μl of each sample was placed in triplicate wells on a microplate, and intensity was measured at 570 nm. Catalase activity was calculated based on the amount of hydrogen peroxide broken down over time and expressed as milligrams of protein or millilitres of sample. Differences in catalase activity between control and experimental groups were plotted as mean fold change.

Statistical Analysis

All the experiments were performed in biological triplicates (at least three fish of each group was used) as well as technical triplicates. The activity of enzymes was presented as mean fold change (mean ± SE). For statistical analysis, student t-test was used and p- value < 0.05 was considered statistically significant.

Result

Determination of LC_{50} Value

To evaluate the biological impact of arsenic contamination, controlled laboratory experiments were conducted using *H. fossilis*. The 96 hr LC_{50} of dibasic sodium arsenate heptahydrate was calculated to be 48.80 mg L^{-1} (Table 1 and Figure 1).

Table 1: Dose-Response Relationship and Probit Analysis of Arsenic Toxicity in *H. fossilis*

Group	Conc. (mg L^{-1})	Log Conc.	No. of Fish	Dead Fish	% Mortality	Corrected % Mortality	Probit Value
Control	0	0.00000	10	0	0	2.5	3.04
Group 1	10	1.00000	10	0	0	2.5	3.04
Group 2	20	1.30103	10	1	10	10	3.42
Group 3	40	1.60206	10	3	30	30	4.48
Group 4	60	1.77815	10	6	60	60	5.25
Group 5	80	1.90309	10	7	70	70	5.52
Group 6	100	2.00000	10	10	100	97.5	6.96

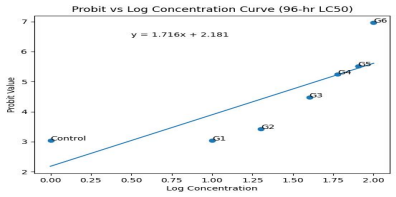

Figure 1: Determination of LC_{50} value of dibasic sodium arsenate heptahydrate. Probit Value 5 equals to 50% mortality of Fish which equals to log concentration 1.6884 or 48.80 mg L^{-1}

SOD Activity Assay

The activity of the antioxidant enzyme superoxide dismutase was analyzed in *H. fossilis* to assess biochemical responses under arsenic exposure at different concentrations and exposure durations. At 96 hour of exposure (fig 2A), a marked variation in SOD activity was observed between the two experimental groups. In fishes exposed to Conc. 1, SOD activity showed a substantial increase of approximately 3.5-fold compared to the control group. The increase was consistently recorded across the sampled individuals, indicating a measurable elevation in enzyme activity at this exposure level. In contrast, fish exposed to Conc. 2 did not exhibit any noticeable change in SOD activity, and the recorded values remained comparable to those of the control group. The enzyme activity values for both experimental groups and the control were measured under identical laboratory conditions, and the results were tabulated accordingly. The graphical representation provided in Figure 2 illustrates the difference in SOD activity between control and treated groups at 96 hour, clearly showing the variation between the two concentrations.

At 7 days of exposure (fig 2B), further changes in SOD activity were recorded in both experimental groups. In fishes exposed to Conc. 1, SOD activity remained elevated, showing an increase of approximately 1.8-fold compared to the control group. Although the increase was lower than that observed at 96 hour, the enzyme activity continued to be higher than baseline levels. In the case of Conc. 2, SOD activity showed a measurable increase of approximately 1.4-fold relative to the control group. This indicates that, over the extended exposure period, both concentrations resulted in observable changes in enzyme activity. The recorded values for each group at 7 days were documented and the corresponding trends are illustrated in Figure 2.

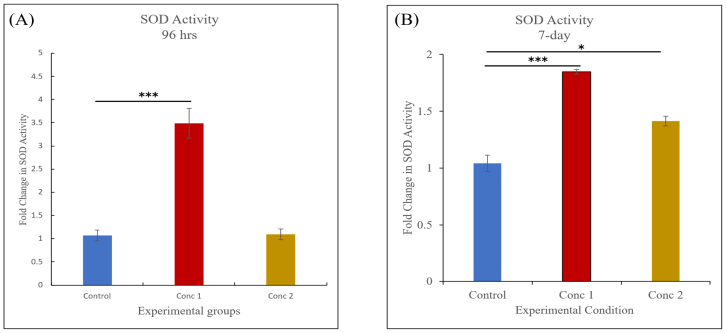

Figure 2: Comparison of SOD activity between control and treated groups at 96 hours (A) and 7-day exposure periods (B)

Catalase Activity Assay

The activity of the antioxidant enzyme catalase was evaluated in *H. fossilis* to assess biochemical responses under arsenic exposure at different concentrations and exposure durations. Two sub-lethal concentrations, namely Conc. 1 (10% of LC_{50}) and Conc. 2 (30% of LC_{50}), were selected for the study. Observations were recorded at two exposure intervals, 96 hour and 7 days.

At 96 hour of exposure (Figure 3A), a marked increase in catalase activity was observed in both treated groups compared to the control. The control group exhibited baseline activity close to 1-fold. In contrast, fish exposed to Conc. 1 showed a substantial increase in catalase activity, reaching approximately 2.0-fold relative to the control. Similarly, the Conc. 2 group also demonstrated an elevated enzyme activity of around 1.9-fold. The increase observed in both treatment groups were statistically significant when compared with the control, indicating a strong enzymatic response under arsenic exposure at this time point.

At 7 days of exposure (Figure 3B), catalase activity remained elevated in both experimental groups, although the magnitude of increase was lower than at 96 hour. The control group maintained a baseline enzyme activity (∼1-fold). Both Conc. 1 and Conc. 2 groups exhibited a significant increase of approximately 1.2-fold compared to the control.

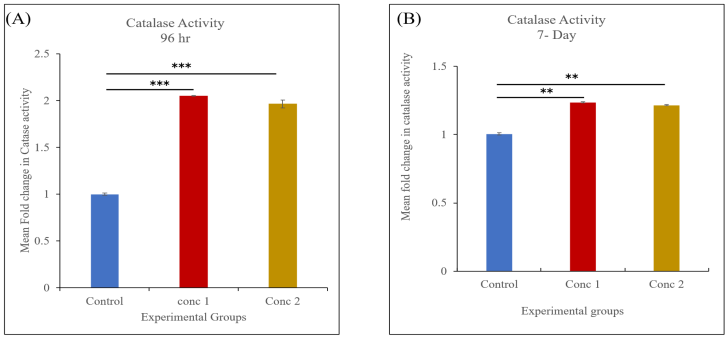

Figure 3: Comparison of catalase activity between control and treated groups at 96 hr (A) and 7-day exposure periods (B)

Discussion

Biochemical Responses and Oxidative Stress

Alterations in antioxidant enzyme activity, particularly superoxide dismutase, constitute a critical biochemical response to arsenic exposure and serve as reliable indicators of oxidative stress in aquatic organisms. Elevated SOD activity at lower toxicant concentrations indicates activation of intrinsic defense mechanisms against increased production of reactive oxygen species. The oxidative stress model proposed by Livingstone (2001) suggests that exposure to environmental pollutants, including heavy metals, results in enhanced generation of ROS such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals, which can cause significant damage to cellular macromolecules. Nishikimi et al. (1972) reported that arsenic exposure induces excessive generation of reactive oxygen species, including hydrogen peroxide. Ellmann (1959) and Hebig et al. (1974) also demonstrated altered antioxidant enzyme activity in organisms exposed to arsenic.

The upregulation of SOD activity under moderate stress is an adaptive and protective response because this enzyme catalyses the dismutation of superoxide radicals into less harmful molecules like hydrogen peroxide and oxygen. Similar increases in antioxidant enzyme activity have been widely reported in fish exposed to heavy metals. For example, Ahmed et al. (2008) and Humtsoe et al. (2007) documented significant elevations in SOD activity in fish subjected to metal-induced stress. This suggests that antioxidant defense mechanisms are rapidly mobilized to counteract oxidative damage. However, under higher toxicant exposure, the absence or suppression of SOD activity indicates a different physiological response. This may result

from enzyme inhibition, structural damage to proteins, or excessive ROS production that overwhelms the antioxidant defense system. These conditions lead to oxidative imbalance, where ROS generation exceeds the organism's capacity to neutralize them. This agrees with Das et al. (2012), who reported that high toxicant concentrations can impair antioxidant enzyme activity, causing oxidative stress–induced cellular damage.

Comparison of superoxide dismutase activity across the two exposure durations indicates that enzyme activity varies with both concentration and time. Measurements at 96 hour and 7 days reveal distinct differences in fold change between the two concentrations. Observations were systematically measured, recorded, and tabulated, and the graphical representation clearly illustrates variations in SOD activity under different experimental conditions. The initial increase in SOD activity at 96 hour reflects an early biochemical response to arsenic exposure, whereas the reduced fold changes at 7 days suggest a shift in enzyme activity patterns over time Khalid & Azmat (2025). Additionally, the delayed increase in SOD activity during prolonged exposure implies a time-dependent adaptive mechanism, in which organisms gradually strengthen their biochemical defense systems in response to sustained stress. This adaptive response is consistent with findings by Oruc and Uner (1999), who reported that prolonged toxicant exposure results in compensatory activation of antioxidant enzymes following an initial phase of suppression or imbalance.

The elevated catalase activity at 96 hour indicates an early antioxidant defense mechanism activated in response to arsenic-induced oxidative stress Bhattacharya & Bhattacharya (2007). The reduced fold change at 7 days suggests physiological adaptation or regulation of enzyme activity during prolonged exposure Ray et al. (2017). Both concentrations showed similar trends, indicating that catalase responds consistently to arsenic stress, although the response intensity decreases over time. These findings highlight catalase as an important biomarker for oxidative stress in *H. fossilis* Ray et al. (2017). Overall, comparison across exposure durations suggests catalase activity is influenced by both concentration and exposure time. A pronounced increase at 96 hour followed by a reduced but sustained elevation at 7 days reflects a time-dependent enzymatic response to arsenic-induced oxidative stress Bhattacharya & Bhattacharya (2007) and Ray et al. (2017).

Conclusion

Arsenic exposure induces significant oxidative stress in *Heteropneustes fossilis*, initially triggering antioxidant defense such as superoxide dismutase and catalase to mitigate reactive oxygen species generation. Lower concentrations of arsenic elicit a

more robust adaptive response; however, prolonged exposure diminishes enzymatic protection, resulting in chronic oxidative damage, tissue degeneration, and inhibited growth. These results demonstrate that arsenic contamination presents substantial ecological risks to aquatic organisms and may pose public health hazards through biomagnification in edible fish consumed by humans.

Acknowledgement

We would like to thank Dr. Amresh Kumar Singh, Department of Zoology, BHU, Varanasi, for providing the microplate reader facility.

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Received: 04.06.2026, Revised: 08.06.2026, Accepted: 10.06.2026