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# Neurotoxicity induced by herbicides Almix and Excel Mera 71 in the Indian teleost *Heteropneustes fossilis* (Bloch)

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Population explosion and simultaneous rapid loss of cultivable land escalated the use of pesticides and herbicides to ensure food security. However, uncontrolled application of toxic pesticides/herbicides, particularly organophosphorus and sulfonylurea, has resulted in increased mortality and morbidity worldwide. The present study reports the neurotoxic effect of herbicides Excel Mera 71 and Almix based on brain histopathology of the Asian stinging fish, *Heteropneustes fossilis* (Bloch). Herbicides, Excel Mera 71 (17.2 mg/L) and Almix (66.67 mg/L) were administered to fish for 30 days. Study showed several endpoints in optic tectum with varying degree. Spongiosis, binucleated nuclei, congestion, necrosis, enlarged pyramidal cells (EPC), neuronal degeneration, vacuolization, gliosis, aberrant horizontal cell orientation and granular cells were prominent pathological symptoms in optic tectum in Excel Mera 71 and Almix-treated *H. fossilis*. Among the six layers, SPV (stratum periventriculare) and SAC (stratum album centrale) layer were maximally impacted by herbicide exposure. Appearance of neural necrosis, gliosis, vacuolization and loss of Nissl substances, collectively called neurotoxicity indicators, were very prominent under herbicides treatment. Higher prevalence of these lesions under Excel Mera 71 indicated that this herbicide has higher neurotoxic potential than Almix. Additionally, mean assessment value (MAV) indicated highest pathological lesions in Excel Mera 71 (4.06 ± 0.02), followed by Almix (3.28 ± 0.10) compared with control fish (0.09 ± 0.04). Collectively, these alterations indicated that both herbicides caused adverse effects on brain metabolism including sensory modalities, visual appearances, and motor coordination. Accordingly, brain optic tectum pathological changes can be utilized as biomarker of neurotoxicity evaluating of environmental toxicants.

**Keywords:** Asian stinging catfish, Brain optic tectum, Fossil cat, Herbicides, Neurotoxic, Pesticides, Shingi Fish

Pesticide poisoning is a serious concern of mortality and morbidity worldwide. The World Health Organization attributes over five million severe poisoning and approximately annual 0.2-0.25 million deaths to pesticide poisoning globally every year[1,2]. The organophosphorus (OP) substances, among them, are utilized extensively in cultivation fields, forestry, medicine, public health and industry[2]. Contrarily, the use of sulfonylurea group herbicide in agricultural fields is very less in India as well as world. In addition to their conventional usage in reducing insects, weeds, and other pests, OP pesticides have a negative impact on aquatic organisms that are not their intended targets, such as fish and humans[2,3]. Glyphosate 71 SG (Excel Mera 71), a third-generation herbicide and Almix 20WP, a fourth-generation herbicide, are extensively utilized compounds in Indian agronomy to increase the productivity[4,5].

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Excel Mera 71 is a systemically active organophosphate herbicide. On the other hand, Almix 20WP [(methyl 2-(4-methoxy-6-methyl-1, 3, 5-triazin-2-yl-carbamoylsulfamoyl) benzoate) + (ethyl 2-(4-chloro-6-methoxy-pyrimidin-2yl-carbamoyl-sulfamoyl) benzoate)] is a systemic and contact herbicide in the sulfonylurea category[4,5]. In addition, Excel Mera 71 is a third-generation herbicide while Almix is a fourth-generation herbicide[4,5]. Toxicological effects of different glyphosate formulations to animals including humans have been reported globally[6]. Farmers and their producers, in particular, are the key risk categories for glyphosate and Almix exposure. Literature on adverse effects of commercial glyphosate formulations in animals has focused primarily on physiological, biochemical and molecular changes. Consequently, our previous study demonstrated chronic exposure of Excel Mera 71 caused oxidative toxicity in kidney, gills and liver of *Anabas testudineus* and physiological, haematological and genetic alterations[7,8]. Almix is known to alter physiological/haematological[9] and pathological[10] responses in fish. Furthermore, acute or chronic exposure caused drastic population decline because of their persistent nature in the environment and subsequent bioaccumulation or biomagnification[11]. However, the hazardous responses of Almix and Excel Mera 71 on animal health with particular reference to structural and functional integrity are not much available till today. Additionally, this structural and functional integrity of cells and/or tissue *i.e.*, histoarchitecture is directly correlated with physiological and biochemical alterations[12]. Histoarchitectural study is an integral aspect of evaluating the structural changes caused by xenobiotics and considered as good biomarker of xenobiotic stress. Additionally, pathological studies help to determine the fundamental relationships between biological responses and contaminant exposure[12]. Pathological study on different fish organs is well documented both under field[13] and laboratory reports[7,8,14]. However, there is dearth of available information on pesticide-induced neuronal dysfunction based on brain histopathology[4,15].

The Indian teleost, *Heteropneustes fossilis* (Bloch), commonly called the Asian stinging fish or Fossil cat, is a commercially valuable fish in India. It lives in stagnant freshwater bodies such as muddy ponds, canals, lakes, beels, tanks, chaurs and swamps as a natural inhabitant. Several characteristics, such as easy availability, wide distribution, easy acclimatization and commercial importance, etc., makes it a good experimental aliquot[4]. Generally, fish physiology and neural dysfunction is regulated by brain. The brain's membrane contains comparatively higher polyunsaturated fatty acid (PUFA) content than other organs/tissues, making this more sensitive to xenobiotics. Furthermore, the presence of small amount of non-enzymatic and enzymatic oxidant accelerated the phenomenon[11-15]. Thus, brain provides an early warning signal compared with other tissues and considered as an important biomarker of xenobiotic exposure. However, till today no study demonstrated neuronal dysfunction based on brain histopathology caused by Excel Mera 71 and Amix[4,15]. Therefore, in the present study, we investigated the adverse effects influenced by Excel Mera 71 and Almix exposure based on brain histopathological observations in *H. fossilis*. Furthermore, we added neuronal dysfunction, apoptosis and neurotoxic potential assessment of Excel Mera 71 and Almix in the current investigation.

## Materials and Methods

### Experimentation procedure

A fish model was established as previously stated research[4,5]. *Heteropneustes fossilis* (mean body weight: 30.67 ± 2.56 g; length: 15.58 ± 0.56 cm) was obtained from an aquarium specialised fish farm and housed in research tanks in consistent oxygenation (250 L). For 15 days, *H. fossilis* were acclimatised to dechlorinated tap water (temperature: 25.21 ± 0.21°C; electrical conductivity: 388.22 ± 0.56 µS/cm; pH: 7.64 ± 0.05; total dissolved solids: 276.24 ± 0.66 mg/L and dissolved oxygen: 6.69 ± 0.06 mg/L). Throughout acclimatisation and experiments, the 12 h light/ darkness was followed, and commercialized pellets (32% crude protein, Tokyu) were supplied daily. The fish tank water was refreshed every two days during acclimatisation, and 2% death was detected.

### Chemicals

This investigation used two herbicides: Almix (purity 98%) and glyphosate-based Excel Mera 71 (purity 98%). These herbicides were obtained from DuPont India Pvt. Ltd. in Gurgaon, Haryana, and Excel Crop Care Ltd. in Mumbai, Maharashtra, India, respectively. In deionized water, standard herbicide solutions were produced.

### Experimental design

Three categories of fish were used in this study: control; Excel Mera 71 (17.2 mg/L) exposure; and Almix exposure (66.67 mg/L)[4,5,7-10]. Each condition was performed thrice, with six fish per tank. Tank water was replenished every 2 days and kept at a semi-static setting for 30 days. Operating parameters were similar with acclimatization of the fish. The water quality was assessed using APHA guidelines[16] and the experimental design was authorised by the University of Burdwan's Ethical Board.

### Histological study procedure

Histological study was performed according to Samanta & Ghosh[4] technique. Following anaesthesia with MS-222 (Sigma-Aldrich, St. Louis, MO, USA), the fish were dissected at the end of the experiment. To clean artefacts like blood and/or mucus from brain, 0.9% physiological NaCl solution was used. Tissue was then immediately preserved in Bouin's mixture and dehydrated by graded ethanol solution followed by xylene washing and finally embedded in paraffin wax (58-60°C melting temperature). Microtome (Leica RM2255, Wetzlar, Germany) sections were cut at 4-5 µm diameter and sections were H&E stained. Finally, stained slices were inspected at various magnifications under a light microscope (Leica DM2000, Wetzlar, Germany) to identify and score pathological lesions.

Employing the approach given by Pal *et al.*[17] and Samanta *et al.*[18], histological alterations were scored to determine the MAV (mean alteration value). The incidence of histological lesions (MAV) was calculated as follows: absence of lesions (accounts for up to 10% of overall inspection), which is represented by 0, was given the value of 1; seldom seen (11-25% of overall inspection), which is represented by $0^+$, was given the value of 2; present (26-50% of overall inspection), which is represented by +, was given the value of 3; persistent lesions (51-75% of overall inspection), which is represented by ++, was given the value of 4; and highly persistent (76-100% of overall inspection), which is represented by +++, was given the value of 5. Finally, the MAV for each treatment was categorized based on respective numeric value according to Samanta & Ghosh[4]: 0-1.0 represents absence of lesions; 1.1-2.0, modest lesions; 2.1-3.0, moderate changes; 3.1-4.0, numerous alterations and 4.0-5.0 represents wide distribution of lesions.

**Statistical analyses**

The statistical package for the social sciences (SPSS) version 25.0 was used to conduct one-way ANOVA. Additionally, Tukey post-hoc test was run to analyse significant variations ($P$ <0.05) between different conditions. Mean ± standard deviation was utilized to display the data (n = 6).

## Results

Brain optic tectum pathological alterations of *Heteropneustes fossilis* exposed to 30 d Excel Mera 71 and Almix treatments were presented in Table 1. Control brain of *H. fossili*s did not show any visible or discernible changes; six layers are clearly distinguished from outer layers to inner: SM (stratum marginale) followed by SO (stratum opticum), SFGS (stratum fibrosum et grisium superficiale), SGC (stratum griseum centrale), SAC (stratum album centrale), SPV (stratum periventriculare) and stratum (S). Additionally, all six layers are in intact position with each other (Fig. 1).

The brain of exposed fish showed alterations at varying degrees of severity. Figs 2 and 3 showed the most prominent pathological findings in brain of *H. fossilis* under herbicides exposure, and Table 1 outlined the degree of frequency and severity of each pathological finding. Six pathologies were more frequently observed in brain tissue under herbicides exposure: vacuolization and necrosis (often associated with binucleated nuclei); neuronal degeneration (often associated with dying of neurons and neuron dilation); proliferation of pyramidal cells and neurons; gliosis and spongiosis; congestion of layers (often associated with dispersion); layer disconnection from neighbouring one (Figs 2 and 3). In addition to this,

Table 1 — Pathological lesion noticed in brain optic tectum of *Heteropneustes fossilis* exposed to Almix and Excel Mera 71

| Layers | Characteristics | Lesions severity | | |
|---|---|---|---|---|
| | | Control | Almix | Excel Mera 71 |
| SPV | Congestion | 0 | $0^+$ | + |
| | Necrosis | 0 | $0^+$ | + |
| | Vacuolization | $0^+$ | + | +++ |
| | Dispersed granular cells | 0 | $0^+$ | + |
| | Binucleated nuclei | 0 | + | +++ |
| | Vascular dilution | 0 | $0^+$ | + |
| | Spongiosis | 0 | $0^+$ | + |
| | Moving towards TS | 0 | $0^+$ | $0^+$ |
| | Enlarged pyramidal cells | 0 | + | ++ |
| | Granular cell dispersion | 0 | ++ | ++ |
| SAC | Necrosis | 0 | ++ | ++ |
| | Vacuolization | $0^+$ | ++ | +++ |
| | Vascular dilution | 0 | $0^+$ | + |
| | Binucleated nuclei | 0 | +++ | +++ |
| | Enlarged pyramidal cells | 0 | ++ | +++ |
| SGC | Necrosis | 0 | + | + |
| | Vacuolization | $0^+$ | + | ++ |
| | Enlarged pyramidal cells | 0 | $0^+$ | + |
| | Binucleated nuclei | 0 | ++ | +++ |
| | Neuronal degeneration | 0 | ++ | +++ |
| | Gliosis | 0 | $0^+$ | $0^+$ |
| SO & SM | Detachment between two layers | $0^+$ | + | +++ |

[No lesions (lesions up to 10% of total analyzed tissue) is represented by 0; rarely present (lesions ranging 11-25%) is represented by $0^+$; presence of lesions (lesions ranging 26-50%) is represented by +; frequently present (lesions ranging 51-75%) is represented by ++; and highly frequent lesions (lesions ranging 76-100%) is represented by +++]

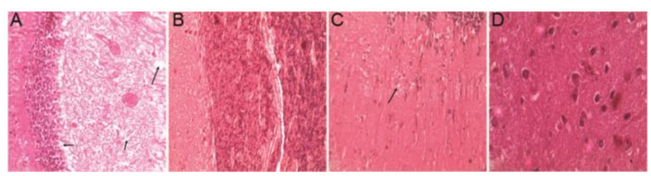


Fig. 1 — Control brain optic tectum of *Heteropneustes fossilis*: (A to D) brain tectum zones are compactly organized (outer to inner) stratum marginale (SM), stratum opticum (SO), stratum fibrosum et grisium superficiale (SFGS), stratum griseum centrale (SGC), stratum album centrale (SAC), stratum (S) periventriculare (SPV). [Vacuolization seen in some places (arrow)]

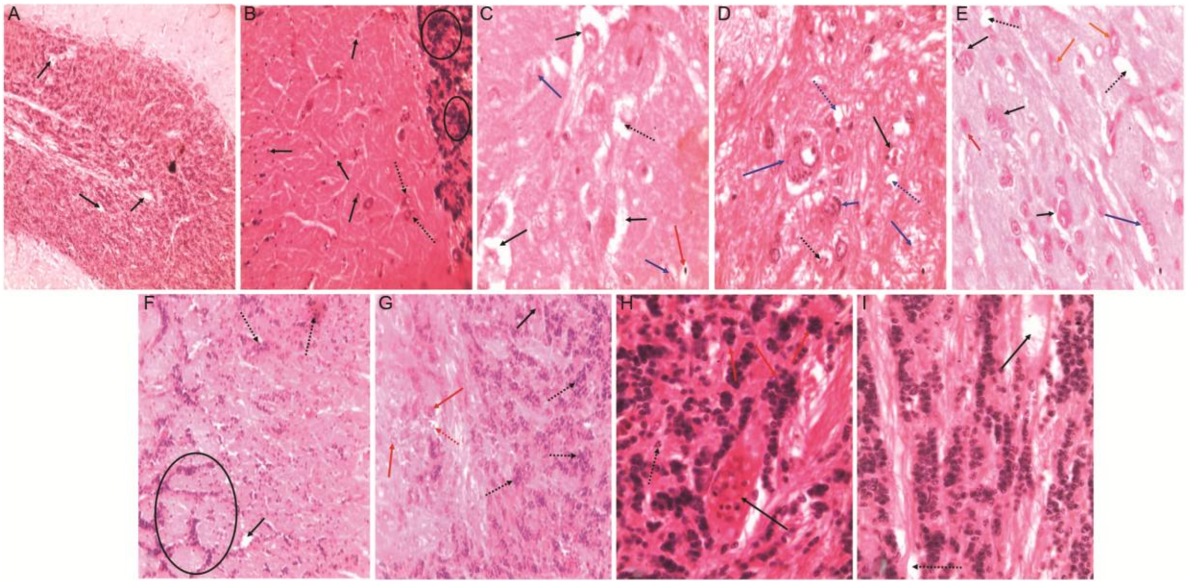


Fig. 2 — Brain pathological lesions in *H. fossilis* exposed to Almix herbicide: (A) vacuolization (arrow); (B) loss or Nissl substances with abnormal sizes (arrow), granular cells clumping (circle), neuronal damage with dilation and vacuolization (broken arrow); (C) degenerative neuron with dilation (arrow), necrosis (broken arrow), dying neuron with mild necrosis (blue arrow), abnormal Nissl substances (red arrow); (D) binucleated nuclei in neurocytes (arrow), pyknotic nuclei (broken arrow), glial nodule formation (blue arrow), vacuolation (broken blue); (E) gliosis (arrow), appearance of vacuole (broken arrow), degeneration in pyramidal cells (blue arrow), dying neuron (red arrow); (F) gliosis (broken arrow), vacuole formation (arrow), dispersed granule cells, lobule formation of granule cells (circle); (G) spongiosis (arrow), clumping of granule cells (broken arrow), mild necrosis (red arrow), binucleated nuclei (red broken); (H) spongiosis (arrow), binucleated nuclei (broken arrow), granular cell clumping (red arrow); and (I) necrosis (arrow), vacuolization (broken arrow).

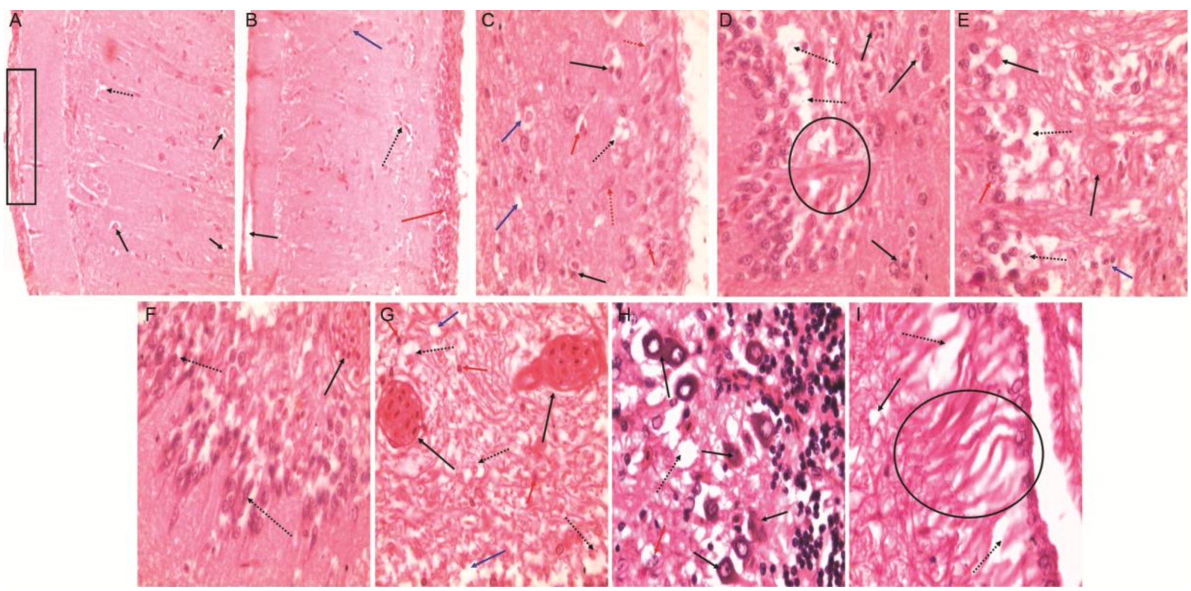


Fig. 3 — *Heteropneustes fossilis* brain lesions exposed to Excel Mera 71: (A) spongiform appearance of SM layer due to vacuolization (box), abnormal Nissl substances (arrow), vacuolization (broken arrow); (B) detachment of SM layer from SO layer (arrow), enlarged pyramidal cells, EPC (broken arrow), dilation in EPC (blue arrow), clumping of granule cells (red arrow); (C) binucleated nuclei in neurocytes (arrow), necrosis in neurones (broken black arrow), unusual Nissl compounds (red arrow), dilated and dying neuron (red broken), vacuolization (blue arrow); (D) gliosis (arrow), severe necrosis (broken arrow), disarrangement in neuronal orientation (circle); (E) spongiosis (arrow), vacuolization and severe necrosis (broken arrow), loss of Nissl substances and neuronal orientation, pyknotic nuclei (red arrow), binucleated nuclei (blue arrow); (F) spongiosis (arrow), clumping and moving of granular cells towards Torus semicircularis (broken arrow); (G) spongiosis (arrow), necrosis in neurones (broken arrow), vacuolization (blue arrow), loss of Nissl substances (red arrow), (H) dying neurons with pyknotic nuclei and nuclear vacuolaion (arrow), vacuolization (broken arrow), peripheral appearances of nuclei in neurocytes (red arrow); and (I) vacuolization (arrow), severe necrosis (broken arrow), loss of neuronal orientation (circle).

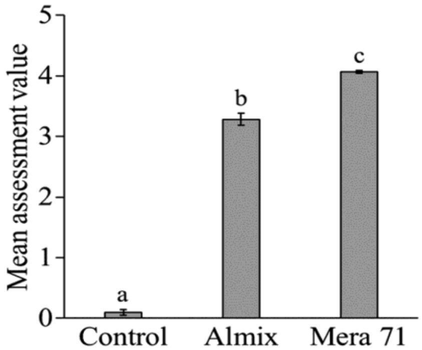


Fig. 4 — *Heteropneustes fossilis* brain MAV level under Excel Mera 71 and Almix intoxication. The values indicated as mean ± standard deviation. Significance level ($P$ <0.05) are shown by alphabets above columns.

Purkinje cells were damaged seriously and neurons showed loss of normal orientation. Among the layers, innermost layers *i.e.*, SAC and SPV were more severely affected in comparison to other layers. Moreover, the degree of severity and prevalence of pathological frequency were much higher (>25%) under Excel Mera 71 exposure compared with Almix exposure (Table 1).

The morphometric index MAV calculated for brain of *H. fossilis* is displayed in Fig. 4. The MAV results revealed considerable disparities between different conditions ($P$ <0.0001). The Almix MAV score was 3.28 ± 0.10, suggesting frequent lesions, whilst the Excel Mera 71 MAV score was 4.06 ± 0.02, implying a wide range of alterations. Additionally, the findings showed significantly higher MAV value in Excel Mera 71 exposure, in comparison with Almix treatment ($P$ <0.0001).

## Discussion

The present study preliminary demonstrated toxic potential and neurotoxic effects of Excel Mera 71and Almix in *H. fossilis* based on pathological investigation. Because xenobiotic exposure is known to exhibit physiological responses primarily mediated by neuroendocrine system[4,15,19]. Brain is the prime centre of containing numerous neurons, serving as relay station as well as controlling all movements and functions[4,15]. Generally, fish brain consists of four regions namely telencephalon, mesencephalon, cerebellum and diencephalon. Histologically, brain is made up of many neurons, glial cells and blood arteries that maintain the CNS cells (central nervous system). Neurons are made up of single cell body with cytoplasm and nucleus, one axon, and one or more dendrites and are distributed in granular, molecular and Purkinje layer[4,15]. However, in the present study, we considered tectum region especially mesencephalon, as this region is homologous with the midbrain of mammalian superior colliculus and most conspicuous dorsal structure of teleost. Additionally, this region controls sensory modalities (somatosensory, audio, electrosensory and lateral line) in addition to visual output/inputs[20,21].

Brain histological alterations of *H. fossilis* clearly indicated cellular reactions to herbicides exposure; the brain tectum under Excel Mera 71 exposure was more adversely affected compared with Almix exposure. Accordingly, the pathological index (MAV) showed significant differences among different treatment conditions with higher values under Excel Mera 71 exposure (Fig. 4). Excel Mera 71-treated optic tectum had MAV value greater than 4, indicating a wide distribution of lesions. However, Almix-treated optic tectum yielded MAV value in the range between 3 and 4, indicating lesions were frequently distributed. Accordingly, in our study we found that Almix and Excel Mera 71 intoxication caused extensive brain tectum pathological alterations which included vacuolization, elongated pyramidal cells (EPC), binucleated nucleus, necrosis, pyknotic nuclei, neuronal degeneration, gliosis, spongiosis, congestion of granular layers, granular cells dispersal, disconnection between layers, *etc.* (Figs 2 and 3). Pesticide intoxication induced similar responses in the tissue architecture of the brain tectum in different teleosts[4,20,21,22]. Vacuolization of the optic tectum nucleus and between layers (SAC, SGC and SPV layers) is indicative of mitochondrial as well as microsomal dysfunction, in particularly due to glycolysis[4,23]. Additionally, this vacuolization indicated neurodegeneration in SAC, SGC and SPV layers[23]. As a result, *H. fossilis's* vacuolization caused a decline in its ability to coordinate its typical motor movements[21,24]. Necrosis, generalized spongiosis and severe congestion of mononuclear and granular cells of SM, SO and SFGC layers indicated impaired visual responses and severe brain damage[4,20,23]. However, the degenerative changes in SGC, SAC and SPV layers, in particularly mononuclear and granular cells indicated apoptosis[4,15,25]. Apoptosis is a type of planned cell death that occurs in tissue remodelling and degeneration[25]; in the present study, it was seen in granular as well as mononuclear cells of SPV, SGC and SAC.

Severe necrosis in combination with vacuolization in neuronal cells of brain optic tectum especially SPV, SGC and SAC stipulated the presence contaminant's neurotoxic property *i.e.*, Excel Mera 71 and Almix[4,23]. Because one of the key neurodegenerative mechanisms is vacuolization and neuronal necrosis in optic tectum[23,25]. Furthermore, substantial necrosis in the *H. fossilis* brain optic tectum suggested the disappearance of Nissl chemicals and glial cell reaction evident in this study was also corroborated by Patnaik *et al*.[23], who intoxicated sub-lethal cadmium and lead to *Cyprinus carpio communis* L. Moreover, the disappearance of Nissl materials and glial cell response (gliosis), as revealed by the appearance of glial nodules, demonstrated that both Excel Mera 71 and Almix are neurotoxic[4,23,26,27]. Excel Mera 71 has a stronger neurotoxicity potential than Almix, as *H. fossilis* treated with Excel Mera 71 had a greater prevalence and incidence of neuronal degeneration, vacuolization and necrosis. Therefore, the alterations noticed in brain optic tectum of *H. fossilis* might alter normal brain functioning because brain optic tectum regulating optic, motor coordination and other exteroceptive impulses[4,28]. As a result, these changes have an impact on not only visual input and output, but also eye movement and motor coordination[4,20].

## Conclusion

The present findings revealed that herbicides intoxication adversely damaged optic tectum of the Indian teleost, *Heteropneustes fossilis* (Bloch). The degree of severity varied differently under herbicides exposure; higher severity under Excel Mera 71 treatment. Additionally, the findings demonstrated that Excel Mera 71 herbicide have higher neurotoxic potential than Almix. In particularly, SPV and SAC layers were heavily affected in comparison to other layers. Overall, both herbicides might influence brain metabolism of *H. fossilis*, which can be reflected in fish health including growth and reproduction. Moreover, to our knowledge, this study primarily reporting Excel Mera 71 and Almix-induced brain histopathology to assess neurotoxic property. Finally, this work could be used as a starting point for further research into the mechanisms and routes of Excel Mera 71 and Almix-induced toxicity.

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## Conflict of interest

Authors declare no competing interests.

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