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Evaluation of health status of *Oreochromis niloticus* (L.) based on brain optic tectum morphopathology

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Oreochromis niloticus (L.) health status was evaluated based on brain optic tectum morphopathology. *O. niloticus* was exposed to sub-lethal Almix (66.67 mg/L) and Excel Mera 71 (17.2 mg/L) concentrations for 30 d. Results revealed that herbicides exposure caused different morphological changes in brain optic tectum. Almix treatment showed comparatively higher morphological changes compared with Excel Mera 71. The mean assessment value (MAV) showed significantly higher ($p < 0.0001$) value in Almix (4.36±0.09) and Excel Mera 71 (3.60±0.09) exposure, in comparison with control fish (0.09±0.02). Stratum album centrale (SAC) and stratum periventriculare (SPV) were most affected layer compared with others. The observed morphological alterations revealed impaired visual, sensory and motor functions, which ultimately alters brain physiology. In particularly, the existence of necrosis in neural cells, loss of Nissl substances, gliosis and vacuole formations under Almix and Excel Mera 71 exposure indicated neurotoxic nature of these herbicides. However, higher prevalence under Almix treatment indicated higher neurotoxic potential than Excel Mera 71. Therefore, the results presented here are highly significant to toxicologist considering its neurotoxic property. Finally, further studies are needed to explore detailed mode of action of neurotoxicity for adverse outcome pathways (AOP) development at molecular level.

Keywords: Adverse outcome pathways, Almix and Excel Mera 71, Brain, Herbicides, Neurotoxicity, Nile tilapia

The structural and functional integrity of tissue and/or cell is an integral part of regulatory toxicology and normally vary within fairly narrow range defined mainly by metabolism, specialisation and differentiation as well as by metabolic substrates availability and neighbouring tissue/cell constraints[1]. Xenobiotic exposure to tissue/cells can evoke either protective cellular adaptations or pathological changes, which can eventually lead to cell death and ultimately population decline[2-6]. These pathological changes are considered as powerful tool as well as biomarkers to evaluate health status of fish exposed to xenobiotic under both laboratory and field conditions[7-9]. The major advantage of using pathological biomarker over other biomarkers in toxicology is that it allows to evaluate specific target organs toxicity. For example, gills, liver and kidney play prominent role in respiration, accumulation and biotransformation, and excretion of xenobiotic, respectively from fish body[10,11]. Additionally, pathological changes can translate into higher biological levels and provides linkage between lower and higher biological levels to understand the adverse outcomes[1,9]. Moreover, pathological lesions observed in these organs are normally easier to identify than functional ones[11], and serve as an early and sensitive warning responses of xenobiotic damage to animal health[7,9]. Furthermore, pathological responses of xenobiotic exposure resulted ecological changes[2].

Fish brain is the controlling center of all functions, specifically related to sense organs, motor coordination, behaviour and metabolic regulation and movements in the body organisms as well[2,9,12]. Brain contains numerous nerve cells and sensory systems. Higher PUFA (polyunsaturated fatty acid) content and lower enzymatic and non-enzymatic oxidant level compared with other tissues makes it more susceptible to xenobiotic exposure[2]. Consequently, brain is considered as good biomarker of xenobiotic exposure because of its early warning signs. However, pathological responses in fish nervous system, in particularly on neurons and their associated processes such as neurobehavioral characteristic, apoptosis, *etc.*, are least studied. Additionally, only little information is available on the effects of pesticides on brain optic

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tectum, which correlates optic impulses with other exteroceptive impulses[13]. Furthermore, no study has reported the toxic effects of Excel Mera 71 (Glyphosate 71 SG) and Almix 20WP on brain optic tectum of *Oreochromis niloticus* based on pathological observations. Excel Mera 71 (Glyphosate 71 SG) and Almix 20WP are widely used herbicides in Indian agriculture to control insects, weeds or other pests[14-16]. Excel Mera 71 is an organophosphorus herbicide with systematic action while Almix 20WP is a sulfonylurea group herbicide with contact and systematic action. Both herbicides are highly water soluble.

Therefore, in this study we tried to (i) to assess the pathological lesions in brain optic tectum of Nile Tilapia, *Oreochromis niloticus* exposed to Excel Mera 71 and Almix 20WP herbicides; (ii) to analyse pathological responses in fish nervous system, in particularly on neurons and their associated processes such as neurobehavioral characteristic, apoptosis, *etc.*; and (iii) to identify the degree of neuro-toxic potentiality of Excel Mera 71 and Almix 20WP herbicides. *O. niloticus* (L.) is considered as model organism as they grow fast, mature quickly, breed easily, acclimatize quickly, have good cultivation potential and considerable commercial importance[17,18]. Further, it is a surface feeding omnivorous fish belonging to Cichlidae family.

Materials and Methods

Chemicals and experimental animal

Two commercial herbicides, Excel Mera 71 (Glyphosate 71 SG) and Almix 20WP were selected for this study. Excel Mera 71 and Almix were purchased from Excel Crop Care Ltd. (Mumbai, Maharashtra, India) and DuPont India Pvt. Ltd. (Gurgaon, Haryana, India), respectively. Excel Mera 71 and Almix 20WP stock solutions were prepared in distilled water.

Omnivorous fish, *Oreochromis niloticus* (mean body weight, 46.38±6.78 g; mean body length, 12.98±0.76 cm) was purchased from specialized teleostean fish farm and maintained under continuous aeration in laboratory tanks (250 L). Fish were acclimatized for 15 d using dechlorinated tap water (temperature: 24.87±0.19°C; pH: 7.44±0.15; electrical conductivity: 408.32±0.68 µS/cm; total dissolved solids: 296.74±1.26 mg/L, dissolved oxygen: 6.78±0.11 mg/L; total alkalinity: 258±8.64 mg/L as $CaCO_3$ and total hardness: 176.46±4.34 mg/L as $CaCO_3$). Aquarium water was replaced on every 2 d and two percent mortality was observed during acclimatization. Fish were acclimatized under natural photoperiod (12 h light-12 h dark) and fed commercial pellets daily (32% crude protein, Tokyu) throughout acclimatization and during experimentation.

Experimental design

To assess the pathological lesions of Excel Mera 71 and Almix 20WP on brain optic tectum of *Oreochromis niloticus*, fish were exposed to Almix treatment (66.67 mg/L) and Excel Mera 71 (17.2 mg/L)[19]. The dose was 1/5th of previously determined LC_{50} value of each herbicide[20,21]. Each treatment was run as triplicate and each tank contains 6 fishes. Simultaneously, a control set was run in triplicate without herbicides. Pathological lesions were compared with control fish. Likely as mentioned above, water was replaced every 2 days and maintained under semi-static conditions for 30 days. Other experimental conditions were consistent with fish acclimation. Water parameters were measured following the methods described by APHA[22]. Fish maintenance and experimental design was approved by the Ethical Committee of the University of Burdwan.

Histological study

Pathological study was conducted according to the methodology described by Ghosh *et al*.[23]. At the end, fish were collected from treated as well as control groups and dissected after anesthetization using MS-222 (Sigma-Aldrich, St. Louis, MO, USA). Brain tissue was collected and rinsed with physiological saline solution (0.9% NaCl) to remove mucus, blood and debris. Brain tissue was then fixed in Bouin's solution and dehydrated using different ethanol concentration. Tissue was then processed for embedding in paraffin wax (melting point 58–60°C) after cleaning in xylene. Leica microtome (Leica RM2255, Wetzlar, Germany) was used to cut brain sections at 4–5 µm thickness and stained with haematoxylin–eosin (H & E). Pathological lesions were identified and examined under light microscope (Leica DM2000, Wetzlar, Germany) at different magnifications. Finally, pathological lesions were scored following the methodology described by Roy et al.[13], Pal et al.[8] and Xing et al.[9] as follows: 0, no lesions (lesions up to 10% of total analyzed tissue); 0^+, rarely present (lesions ranges from 11% to 25%); +, present (lesions ranges from 26% to 50%); ++,

frequent (lesions ranges from 51% to 75%) and +++, highly frequent lesions (lesions ranges from 76% to 100%). Accordingly, a numerical value was given for each lesion namely 1, 2, 3, 4 and 5, respectively to calculate the mean alteration value (MAV) irrespective of fish length. The observed pathological lesions were considered as basis for MAV calculation. The MAV for each treatment was calculated according to Paulino *et al.*[24] based on respective numeric value: 0–1.0, no pathological alterations; 1.1–2.0, focal mild alterations; 2.1–3.0, moderately spread lesions; 3.1–4.0, frequent lesions and 4.0–5.0, widely distributed lesions.

Statistical analyses

Results are represented as mean±standard deviation. One-way variance analysis followed by Tukey test was performed using statistical package (SPSS software, version 25.0) to determine differences at $P < 0.05$ significance level.

Results

The brain optic tectum is comprising of six distinct layers and differentiated with each other compactly. Typical brain optic tectum of fish arranged (inner to outer layer) as stratum (S) followed by periventriculare (SPV), stratum album centrale (SAC), stratum griseum centrale (SGC), stratum fibrosum et grisium superficiale (SFGS), stratum opticum (SO), and stratum marginale (SM). Brain of *O. niloticus* from control group exhibited no morphological lesions (Fig. 1). In contrast, the brain optic tectum of Almix and Excel Mera 71-treated fish showed numerous morphological lesions in different layers of brain. In general, the morphological alterations were more pronounced and severe in Almix-treated fish brain compared with Excel Mera 71-treated fish brain (Table 1). Moreover, the morphological lesions were widely distributed in Almix treatment, whereas under Excel Mera 71 exposure lesions were frequently distributed.

The brain optic tectum of Almix and Excel Mera 71 treated *O. niloticus* showed numerous morphological alterations in different layers. Table 1 summarizes the frequency and degree of severity of each morphological alteration in brain optic tectum of *O. niloticus*. The most frequent alterations observed in

Table 1 — Morphological alterations found in brain optic tectum of *Oreochromis niloticus* exposed to Almix and Excel Mera 71

Layers	Characteristics	Lesion's severity		
		Cont.	Almix	Excel Mera 71
SPV	Congestion	0	0^+	+
	Necrosis	0	+++	++
	Vacuolization	0^+	+++	++
	Dispersed granular cells	0	++	+
	Binucleated nuclei	0	++	++
	Vascular dilution	0	+	++
	Spongiosis	0	++	+
	Moving towards TS	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	++	+
	Binucleated nuclei	0	++	++
	Neuronal degeneration	0	+++	+++
	Gliosis	0	++	+
SO & SM	Detachment between two layers	0^+	+++	+++

[Note: 0, no lesions (lesions up to 10% of total analyzed tissue); 0^+, rarely present (lesions ranges 11-25%); +, present (lesions ranges 26-50%); ++, frequent (lesions ranges 51-75%) and +++, highly frequent (lesions ranges 76-100%)]

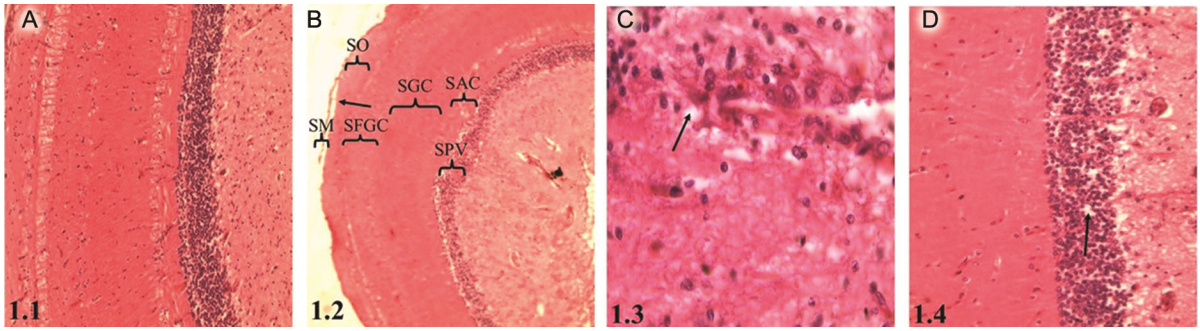

Fig. 1 — Photograph showing control *Oreochromis niloticus* brain optic tectum: (A & B) intact tectum layers (inner to outer) stratum (S) periventriculare (SPV), stratum album centrale (SAC), stratum griseum centrale (SGC), stratum fibrosum et grisium superficiale (SFGS), stratum opticum (SO), and stratum marginale (SM). Detachment of SM from SO (arrow) 400X and 100X, respectively); and (C & D); Appearance of vacuoles (arrow) 1000X and 400X, respectively.

brain under Almix and Excel Mera 71 exposure were binucleated nuclei, enlarged pyramidal cells (EPC), congestion as well as dispersion of granular cells, vacuolization, vascular dilation, neuronal degeneration (including neurons dying, loss of normal orientation and neurons proliferation), necrosis, spongiosis, gliosis, detachment of one layer from another (Figs 2 & 3). Additionally, the Purkinje cells were adversely affected. In particularly, the SPV and SAC layers were damaged more seriously compared with other layers. Collectively, the brain under Almix treatment exhibited higher prevalence of morphological alterations than that of Excel Mera 71-treated brain. These lesions were 25% greater for Almix than Excel Mera 71 exposure.

The mean values of MAV index calculated for brain of *O. niloticus* exposed under Almix than Excel Mera 71 treatments are presented in Fig. 4. Additionally, the values were significantly differed from each other (P <0.05), with lesions ranging from frequent to wide distribution across different layers of brain. In particularly, the index value was significantly higher (P <0.05) in Almix treatment than Excel Mera 71 exposure. The MAV index value for Almix treatment was 4.36±0.09, indicating widely distributed lesions, whereas MAV value for Excel Mera 71 treatment was 3.60±0.09, indicating frequent distribution of lesions.

Discussion

Morphometric analysis in fish are powerful indicators of exposure to environmental stressors in aquatic toxicology. Stressors are known to affect fish's physiology, as like other vertebrates' fish exhibits physiological responses mainly governed by neuro-endocrine pathway[2]. However, the usefulness of morphometric analysis in neurotoxic injury assessment are very scarce[8,25]. Additionally, neurotoxic injury associated with brain histopathology to Almix and Excel Mera 71 exposure have not yet been reported in Indian teleost, *O. niloticus*; it is first of kind to our best knowledge. Therefore, Almix and Excel Mera 71-induced neurotoxic pathology in brain optic tectum of *O. niloticus* reported here are highly relevant and significant to establish the baseline data. The brain optic tectum is highly developed neural processor, controls all functions mainly sensory modalities, body movements and behavioural responses required for survival and reproduction[25]. Our morphometric analysis revealed that *O. niloticus*

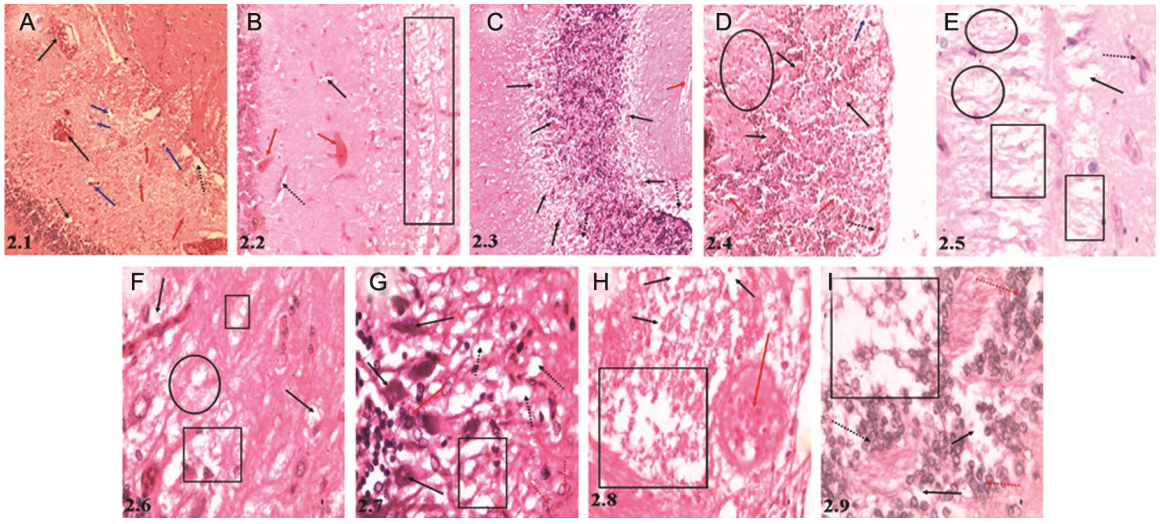

Fig. 2 — Severity of histological lesions in *O. niloticus* brain tectum under Almix exposure: (A) spongiosis (arrow), vacuolization (broken arrow), pyknotic nuclei (blue arrow), abnormal Nissl substances (red arrow) 400X; (B) SFGS and SO disorganized with large vacuoles causing spongiform appearance (box), enlarged pyramidal cells, EPC (arrow), dilated vesicles (broken arrow), dying neurons (red arrow) 400X; (C) dispersion of granular cells, neuronal necrosis with severe vacuolization and dilation (arrow), vacuolization (broken arrow), dilated vessels (red arrow) 400X; (D) spongiosis (arrow), severe damage in granular monolayer, granular cell dispersion (circle), enlarged EPC (broken arrow), severe vacuolization (red arrow), severe necrosis in granular monolayer (blue arrow) 400X; (E & F) severe necrosis in neural cells (square), neuronal disorganization (circle), severe vacuolization (arrow), severe necrosis in vessels (broken arrow) 1000X each; (G) dying neurons (arrow), severe vacuolization (broken arrow), neural necrosis (square), gliosis (red arrow), degenerated nuclei (red broken) 1000X; and (H & I) severe necrosis (square), severe vacuolization (arrow), spongiosis (red arrow), gliosis (broken arrow), clumping of granular cells (red broken) 1000X each.

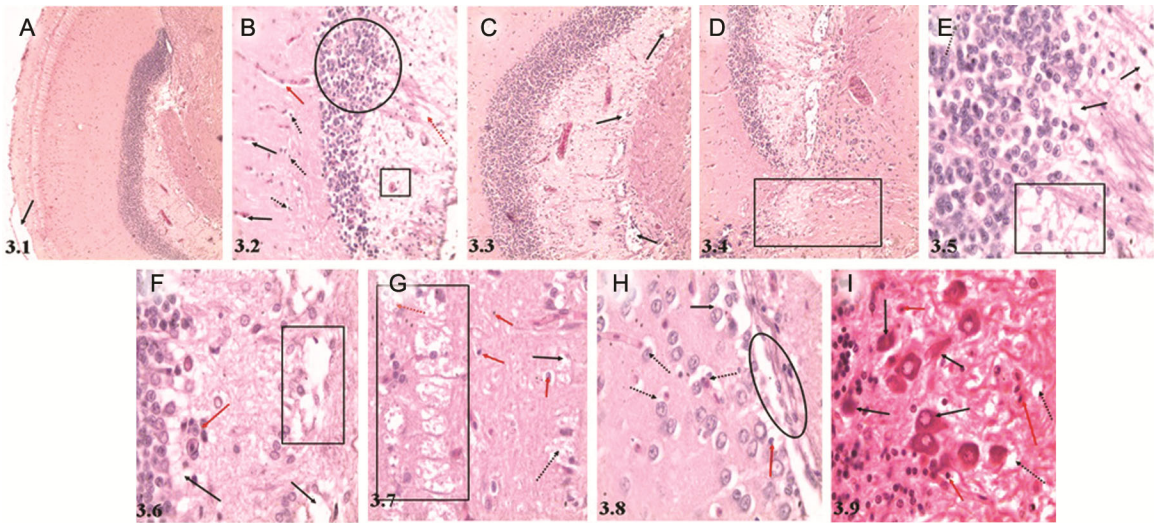

Fig. 3 — Severity of histological lesions in *O. niloticus* brain tectum under Excel Mera 71 exposure: (A) detachment of SM from SO layer (arrow) 400X; (B) dilated vesicles (arrow), abnormal Nissl substances (broken arrow), appearances of space around vesicles (red arrow), dilated neurons (square), glial inflammation (circle), vacuolization (red broken), dispersion of granular cells 400X; (C) vacuolization (arrow) 400X; (D) dispersion of granular cells, disorganization in neuronal cells (square) 400X; (E & F) necrosis (square), vacuolization (arrow), binucleated nuclei (broken arrow), neuronal disorganization, gliosis (red arrow) 1000X each; (G) SFGS and SO disorganized with large vacuoles causing spongiform appearance (box), vacuolization (arrow), necrosis (broken arrow), abnormal Nissl substances (red arrow), fragmented nuclei (red broken) 1000X; (H) glial nodule formation (arrow), degenerative disorder (broken arrow), pyknotic nuclei (red arrow), neuronal degeneration (circle) 1000X; and (I) dying neurons with nuclear vacuolaion and pyknotic nuclei (arrow), vacuole formation (broken arrow), abnormal Nissl substances (red arrow) 1000X

exposed to Almix and Excel Mera 71 treatments negatively affected the brain physiology. The morphometric changes observed in brain optic tectum under present study were similar to typical morphometric responses to other pollutants of previous studies[8,24].

In the present study, the morphometric analysis of brain optic tectum revealed that *O. niloticus* from Almix and Excel Mera 71 treatments exhibited enhanced pathological lesions, in comparison with control fish (Figs. 1-3). Additionally, the morphometric analysis showed that the lesions ranged from frequently distribution to wide distribution in different layers and are indicative of severe brain damage. In particularly, the morphometric analysis displayed that Almix exposure caused higher pathological lesions (wide distribution of lesions *i.e.*, MAV greater than 4), in comparison with Excel Mera 71 exposure (frequent distribution of lesions *i.e.*, MAV ranged between 3 and 4). High incidence of pathological lesions under Almix exposure is an evidence of higher toxic potential to brain optic tectum of *O. niloticus*, in comparison with Excel Mera 71 exposure. Consequently, the morphological changes recorded in brain optic tectum of *O. niloticus* can be attributed to physiological changes in brain and/or other cells[26]. Neuronal degeneration,

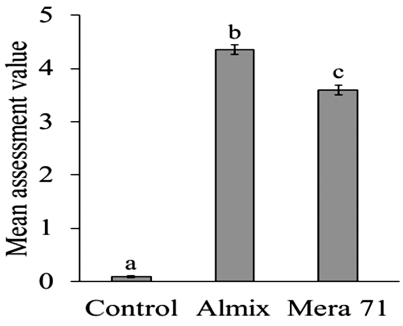

Fig. 4 — Mean assessment value (MAV) in the brain optic tectum of *O. niloticus* exposed to Almix and Excel Mera 71 herbicides. [Data represented as mean±standard deviation. Different letters above the columns indicate significant differences (P <0.05)]

vacuolization, necrosis, gliosis, loss of Nissl substances, spongiosis, congestion of granular layers, enlarged pyramidal cells (EPC), binucleated nuclei, Purkinje cell damage, pyknotic nuclei, dispersion of granular cells, detachment between two layers, *etc.* observed under present study were also noted in different layers of brain optic tectum of fish species exposed to environmental stressors[2,9,25,27]. Neurodegeneration observed in different layers of brain leads to development of clear spaces (SGC, SAC and SPV) between layers and around the nucleus, and vacuolization[25]. Contrarily, the

degeneration in mononuclear and granular cells of SGC, SAC and SPV layers leads to development of apoptosis, which is characterized by programmatic cell death in highly conservative manner[28]. These alterations in deep layers of brain optic tectum impaired motor coordination of *O. niloticus*[25,29].

Vacuolization in different layers of brain tissue might be due to glycolysis leading to mitochondrial and microsomal dysfunctions. In addition, the observed vacuolization in SGC, SAC and SPV layers indicated the existence of neurodegenerative symptoms in these layers[2,25]. Subsequently, vacuolization caused motor impairment and disturbance in relay mechanism in *O. niloticus*[27]. On the other hand, severe congestion, necrosis and generalized spongiosis in SM, SO and SFGC layer's mononuclear and granular cells is an evidence of altered visual responses and severe damage in brain layers[2,25]. Necrosis in neuronal cells of optic layers under Almix and Excel Mera 71 exposure indicated neurotoxic property of these two herbicides[2]. In addition, neuronal necrosis in brain optic tectum indicated loss of Nissl substances[30]. Similarly, Mishra and Devi[25] demonstrated loss of Nissl substances due to presence of neuronal necrosis in exposed *Channa punctatus* to acute and sub-chronic chlorpyrifos. Glial cell reaction *i.e.*, gliosis, evidenced as glial nodule formation in brain optic tectum regions, was another proof of existence of neurotoxic property of Almix and Excel Mera 71[2]. The glial cell reactions as noticed in present study was also reported by Mishra and Devi[25] in *Channa punctatus* exposed to chlorpyrifos. Additionally, they opined that both glial cell reaction and loss of Nissl substances are evidenced as one of the major mechanisms of neurodegeneration. Furthermore, Sarma et al.[2] demonstrated that neuronal degeneration and glial cell reaction are indicative of neurotoxic nature of environmental stressors. However, in this study, higher prevalence and severity of neural degeneration, necrosis and vacuolization in brain optic tectum under Almix treatment indicated that this herbicide has higher neuro-toxic capability compared with Excel Mera 71 exposure. Therefore, these alterations indicated that Almix and Excel Mera 71 exposure adversely impacted brain's normal physiological functions including sensory modalities and behavioural responses[2]. Consequently, these alterations affect not only visual impulses but also motor coordination due to disturbances in blood-brain barrier[2,25].

Conclusion

The present study investigated the neuro-toxic potentiality of Excel Mera 71 and Almix 20WP herbicides using *Oreochromis niloticus* brain optic tectum histopathology. The findings indicated that fish brain is highly sensitive to environmental stressors namely Almix and Excel Mera 71. Additionally, among the two studied herbicides the effects of Almix are more pronounced, in comparison with Excel Mera 71. The results also indicated that both herbicides have neurotoxic property and adversely affected the sensory, visual, and motor functions of fish and ultimately brain physiology. Further, the results suggested that Almix is highly toxic to *O. niloticus*. Thus, dysfunction of brain physiology and neuronal degeneration can serve as a key of Almix and Excel Mera 71 toxicity. This information should be used to explore the mechanistic pathways of Almix and Excel Mera 71 in future studies. Finally, its application in agricultural fields should be judicially operated to avoid contamination of freshwater bodies.

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Conflict of interest

Authors declare no competing interests.

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