

Ameliorative Potential of Astaxanthin on Glyphosate-Induced Neurocognitive Functional Changes in Adult Zebrafish Via Acid Phosphatase and Annexin A2 Dysfunction

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ABSTRACT

Glyphosate (GYP) is a phosphonate type of herbicide and potentially causes neurodegenerations. Astaxanthin (AST) is a red pigment and is a group of carotenoids. It possesses potential antioxidants and neuroprotective actions. However, the ameliorative role of AST in GYP-induced neurocognitive dysfunction has not been studied yet. Hence, the present study is designed to investigate the ameliorative potential of AST in GYP-induced cognitive dysfunction via regulations of acid phosphatase (AP) and annexin A2 (AXA2) actions in the zebrafish model. The cognitive dysfunction was induced by exposure to GYP (0.5 mg/L for 30 minutes/day) for 14 consecutive days. The AST (50, 75, and 100 mg/L for 30 minutes/day) and reference drug i.e., donepezil (DP; 1 µg/mL for 30 minutes/day) were exposed for 14 consecutive days before GYP exposure. On the 14th day, cognitive changes i.e., three horizontal compartment test, optokinetic motor response (OMR), startle response (SR), and T-maze tests were assessed. The changes of biomarkers i.e., acetylcholinesterase (AChE) activity, thio barbituric acid reactive substances (TBARS), reduced glutathione (GSH), AP, and AXA2 levels were estimated in brain tissue. The administration of AST ameliorates the GYP-induced neurocognitive dysfunctions via the antioxidant, regulation of AP and AXA2 actions. Hence, it can be used for neurocognitive disorders against the phosphonate type of herbicide (GYP) toxicity.

Keywords: Acetylcholinesterase; Lipid Peroxidation; Optokinetic Response; Reduced Glutathione; Startle Response Test; Three Horizontal Compartment Test; T-Maze Test

Abbreviations: AP: Acid Phosphatase; TBARS: Thio Barbituric Acid Reactive Substances; SR: Startle Response; OMR: Optokinetic Motor Response; BBB: Blood-Brain Barrier; STAT-3: Signal Transducer and Activator of The Transcription 3; GABA: Gamma-Aminobutyric Acid; ELISA: Enzyme-Linked Immunosorbent Assay; TSLs: Time Spent in The Lower Segment; RPM: Revolutions Per Minute

Introduction

Glyphosate (GYP) is a non-selective phosphonate type of herbicide (Commercially named as Roundup®) [1]. GYP is commonly used in agricultural forms to kill weeds. It is also known as a forever chemical due to mixing in soil, water, and food; and retains a longer period [2]. Globally, about four billion pounds of GYP is used every year [3]. The incidences of GYP toxicity are rising every year [4]; it causes toxicities in the gut, heart, kidney, and reproductive organs including the neuronal system [5]. In the neuronal tissue, GYP enhances neuronal

excitation and loss of neuronal plasticity leads to neurodegenerations [6]. Furthermore, it affects cellular metabolism and causes mitochondrial dysfunctions via free radical generation, release of pro-inflammatory cytokines, and enhance the synthesis & release of glutamate molecules [7,8]. Subsequently, it also causes the dysfunction of the blood-brain barrier (BBB) [9]. Glutamate excitotoxicity is raised with AP activity [8,10]. Moreover, the glutamate-associated membrane translocation of AXA2 is a major factor in the development of abnormal neuronal firing (excitotoxicity) [11]. These processes are also

known to enhance neurodegeneration-associated cognitive disorders like Alzheimer's disease and vascular dementia [12-14]. Our previous report revealed that, GLY causes the cognitive dysfunction via anti-oxidant and acetylcholinesterase inhibitory actions [15]. Besides, the recent report evidenced that, GYP inhibits of hippocampal long-term potentiation and cause the memory dysfunctions [16]. Astaxanthin (AST) is a xanthophyll carotenoid red pigment and it is found in marine microorganisms and animals [17]. It possesses potential antioxidants and neuroprotective actions. It is known to protect tissue injury against various drug and chemical toxicities [18-21]. Moreover, AST ameliorates Alzheimer's disease-related blood-brain barrier dysfunction via clearance of amyloid beta proteins, enhances autophagy, and exerts potential anti-inflammatory actions in *in-vitro* models [22]. Experimentally, it has been proved that AST can reduce neuroinflammation and prevent neurodegenerations [23].

Besides AST also attenuates the aluminum chloride-associated spatial memory dysfunctions and oxidative stress in mice [24]. Similarly, it protects the neuronal tissue against cerebral ischemia/reperfusion-induced neuronal damage in mice [25]. Furthermore, it also regulates the neurovascular unit against streptozotocin-induced diabetic retinopathy via inhibition of neuron-specific enolase actions [26]. Moreover, it reduces the neuroinflammatory actions and memory dysfunctions via the inactivation of signal transducer and activator of the transcription 3 (STAT-3) pathway [27]. AST decreased the tartrate-resistant acid phosphatase activity in periodontitis-associated osteoclast dysfunctions [28]. Normally, AXA2 is expressed in gamma-aminobutyric acid (GABA) interneurons in normal adult brains of rats [29]. The degradation of AXA2 is evidenced to cause dopaminergic neuronal death via regulating the p53 proteins in Parkinson's disease i.e., neurodegenerative disorder [30]. The administration of recombinant AXA2 is evidenced to improve neurological functions against traumatic brain injury in mice [31]. Besides the deficiency of AXA2 causes neuroinflammation and long-term neurological dysfunctions in traumatic brain-injured mice [32]. However, the administration of AST enhances hippocampal neurogenesis (neuronal sprouting) and enhances spatial memory functions in adult mice [33]. In contrast, the deficiency of microglial AXA2 suppresses the neuronal inflammatory responses in mice models of ischemia reperfusion-induced cerebral injury [34]. Further, the upregulation of AXA2 regulates the microglia activations and downstream of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway [34]. However, the role of AST actions on acid phosphatase and AXA2 in neuronal tissue and memory functions is not studied yet. Hence, the present designed to investigate the role of AST actions on glyphosate-induced neurocognitive changes in adult zebrafish.

Materials and Methods

Animals

A total of 120 male adult zebrafish (5-6 months old) were employed in the present study. A total of six groups were used in this

study. Each group comprised 22 male adult zebrafish. The adult zebrafishes were used for the assessment of GYP-induced neurocognitive function and biomarker changes. All the groups of animals were maintained in the separate aquarium tanks at a constant temperature i.e., $28 \pm 1^\circ\text{C}$, fixed oxygen supply with aerators. Throughout the experimental period, standard floating food was supplied as fish food. The tap water and tank water qualities were analyzed every two days. The study was approved by the AIMST University Animal Ethics Committee (AUAEC/FOP/2023/03).

Drugs and Chemicals

AST was procured from Merck Sdn Bhd, Selangor, Malaysia. Colorimetric acid phosphatase assay kit and AXA2 enzyme-linked immunosorbent assay (ELISA) kit were procured from Abcam Inc., Boston, United States of America. 1,1,3,3-tetramethoxypropane (TMP), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, donepezil (DP), GYP, p-nitro-phenyl phosphate, and tricaine mesylate were purchased from Merck Sdn. Bhd, Selangor, Malaysia. All the chemical reagents were used as an analytical grade.

Experimental Protocol

Six experimental groups were employed in this study. Each group consists of 20 adult male zebrafish (6 months old). Group 1 served as a normal control group. This group of animals did not have any exposure to GYP, AST, and DP. Group 2 served as GYP-exposed animals. GYP was exposed to zebrafish at the dose of 0.5 mg/L for 30 minutes per day for 14 consecutive days as described method of Bridi et al. [35]. Groups 3 to 5 were served as AST-exposed groups at the dose of 50, 75, and 100 mg/L for 30 minutes/day for 14 consecutive days respectively. AST was exposed 30 minutes before the GYP exposure. Group 6 was served as DP exposed group at the dose of 1 $\mu\text{g}/\text{mL}$ for 30 minutes/day for 14 consecutive days as described method of Giacomini et al. [36]. AST and DP were exposed 30 minutes before the GYP exposure. On the 14th day, all zebrafish were used for the assessment of neurobehavioral tests i.e., T-maze test, and three-compartment tests. The next day, animals were sacrificed and brain tissues for the biomarkers estimation.

Assessment of GYP-Induced Cognitive Behavioral Tests: GYP-induced changes in zebrafish cognitive behavior patterns i.e., three horizontal compartment test, optokinetic motor response (OMR), startle response (SR) and T-maze tests were assessed. The experimental procedures of behavioral tests were described in the following sections.

Three Horizontal Compartment Test: A three-horizontal compartment test is used for the assessment of neurocognitive functions of zebrafish and assessed as described method of Dubey et al. [37] with minor modification of Rishitha and Muthuraman [38]. Briefly, the test chamber consists of 26.5 cm in length, 7.5 cm in width, and 23.5 cm in height. The chamber was divided horizontally into three equal compartments i.e., 7x7x7 cm of height with glass marking (out-

er of the chamber). The graph paper was fixed on one side of the test chamber to assess the fish movement from the front side (via transparent glass). The water level in the test chamber was maintained up to 21 cm. Before the cognitive function test, animals were trained for 60 seconds to be exposed to all three-compartment areas. The next day, the cognitive function was assessed in three horizontal compartment tests as an indication of "time spent in the lower segment" (TSLs) level. If the animal does not prefer to swim in the upper segment while preferring to swim in the middle and/or lower segments indicates a loss of memory. If the animal swims in the upper segment within 15 seconds and decreases the TSLs levels indicated that memory retention capacity was normal and/or enhanced.

Assessment of Optokinetic Motor Response (OMR): The OMR test is one of the methods of visual cognitive functions in zebrafish animals. It is assessed against the moving black and white stripes as described method of Fleisch and Neuhauss [39]. Briefly, the OMR device consists of two circular transparent acrylic drums. The outer circular drum (200 mm in width and 100 mm in height) was fixed with non-transparent black vertical stripes (10 mm in width and 80 mm in height). The transparent inner circular drum (120 mm in width and 100 mm in height) contains the pole (30 mm in diameter) at the center. This drum is also filled with tank water at a depth of 60 mm. The outer chamber was made rotation at 10 revolutions per minute (RPM) with DC motor control. Zebrafish swim against the moving stripes based on the ability of visual cognitive function as described by Ninkovic and Bally-Cuif [40]. Fish movement was observed with the outer drum's moments by clockwise and counterclockwise movement directions. During the experimental period, the device was illuminated with 300 lux light intensity. One day before the experiment, the first visual stimuli were exposed to zebrafish by placing them in the middle chamber for 1 minute. On the day of the experiment, the baseline exposure was allowed for 1 minute without rotation of the outer chamber. During testing period, zebrafish was allowed to swim with rotation (clockwise and anticlockwise application of visual stimuli) of the outer chamber for 1 minute respectively. The visual cognitive function was assessed in the OMR test as an indication of swimming duration (in seconds) level. If the animal swims towards the rotation of the outer chamber, the total swimming duration increases as an indication of enhanced visual memory retention capacity. If the animal does not prefer to swim with visual stimuli indicates a loss of memory due to a reduction of total swimming duration. The illustration of an optokinetic motor device for the assessment of mice's visual cognitive functions was published by Parameswaran et al. [41]. In this study, a minor modification was made by the application of water to support the zebrafish swimming process.

Assessment of Startle Response (SR): An OMR test is one of the sensitive methods for the assessment of cognitive function in zebrafish animals with response to exteroceptive stimuli (flashlight) as described by Kirshenbaum et al. [42] and Burton et al. [43]. Briefly, the SR test device consists of 26.5 cm in length, 7.5 cm in width, and

23.5 cm in height of transparent square chamber. The water level was maintained for a 4 cm depth. Graph paper was covered at the back of the chamber. The front side of the SR device was allowed to record the movement of the animal with a video camera. One day before the testing of cognitive function, fish were exposed to this SR device for 1 minute to acclimatize to the environment. On the day of the experiment, the number of line crossings within the 2 minutes was recorded to quantify the swimming pattern (percentage startle response; %SR) with measurement of several line crossing by application of low-intensity red LED flashlight (300 lux) before and after 1-minute exposures. The SR test was conducted between 8 AM to 12 noon. The percentage startle response was calculated by using the formula:

$$\%SR = \frac{\text{number of line crossings after light exposure}}{\text{number of line crossings before light exposure}} \times 100$$

Assessment of T-Maze Test: The T-maze test is one of the common methods for the assessment of cognitive function in zebrafish animals as described by Colwill et al. [44] with slight modifications by Rishitha and Muthuraman [38]. Briefly, the T-maze device consists of two small arms with dimensions of 10 cm in length, 6 cm in width, and 10 cm in height of transparent glass. Each arm had different colours; one arm was covered with red color tape and another arm was covered with green colour tape. The connecting middle long arm dimensions were maintained as 20 cm in length, 10 cm in width, and 10 cm in height. The end of the outer corner also consists of a home chamber with dimensions of 5 cm in length, 6 cm in width, and 10 cm in height with a non-transparent glass chamber. One day before the experiment, all the animals were allowed to be exposed in each arm to adapt to the T-maze environments and learn the locations. If the animal reached the red side of the arm, the gentle stirring stimuli were applied with a glass rod. If the animal reached in green side of the arm, the reward stimuli were applied by placing floating food. On the day experiment, the animal was placed in the home chamber and noted as a starting point. The goal point was measured to reach the corners of any one of the short arms; it was noted as transfer latency (TL, seconds). The reduction of TL value indicates the enhancement of memory functions whereas the rising value of TL was considered as memory impairments.

Estimation of Biomarkers Levels: After the assessment of all cognitive behavior tests on the 14th day, zebrafish were kept in a home tank. The next day, zebrafish were sacrificed by using tricaine methane sulfonate at the dose of 150 mg/L. Thereafter, the fish brain tissue was harvested as a described method of Tomizawa et al. [45]. The tissue was homogenates with 1 ml of 25 mM of phosphate buffer saline (pH 7.4). Then, the clear aliquot was used for the estimation of the following biomarkers i.e., AChE activity, TBARS, GSH, AP and AXA2 activity, and total protein levels.

Estimation of AChE Activity: The level of brain AChE activity was estimated as described by Ellman et al. [46]. Briefly, 500 µl of the aliquot was mixed with 0.25 ml of 0.001 M of DTNB solution

and incubated at room temperature (37°C) for 10 minutes. The absorbance changes were noted from the spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., California, United States of America). The wavelength was fixed at 420 nm for the recording of absorbance changes. The total AChE activity levels were calculated by using the following formula:

$$AChE\ activity = \frac{\delta O.D \times Volume\ of\ the\ assay}{\epsilon \times mg\ of\ protein}$$

Here, AChE enzyme activity expressed 'n' mole of acetylthiocholine iodide hydrolyzed per minute in mg of protein; the volume of the assay was fixed in 3 ml; $\delta O.D$ expressed the changes of absorbance per minute; epsilon (ϵ) represents the extinction coefficient i.e., 13,600 per mol per centimeter.

Estimation of TBARS Level: The level of TBARS is an indicator of cellular lipid peroxidation and it was estimated as described by Okawa et al. [47]. Briefly, 0.2 mL of aliquot was mixed with 0.2 ml of 8.1% w/v of sodium dodecyl sulfate, 1.5 ml of 30% acetic acid (pH 3.5), and 1.5 ml of 0.8% w/v of thio barbituric acid. The volume of the test tube was maintained at 4 ml with distilled water and incubated at 95°C in a water bath for 1 hour. Thereafter, test tubes were carefully cooled with tap water. Then, a 1:5 ratio of distilled water and 15% v/v of the n-butanol-pyridine mixture were added. Ten minutes later test tubes were centrifuged for 15 minutes with relative centrifugal force i.e., 1372 g. The changes in pink colour chromogen absorbance were analysed by a spectrophotometer device at 535 nm of wavelength. The reference standard was prepared between 0 to 10 nM of TMP solution.

Estimation of GSH Level: The level of GSH is an indicator of endogenous antioxidant marker and it was estimated as described by Ellman [48]. Briefly, 0.5 ml aliquot was mixed with 0.3 M of disodium hydrogen phosphate solution and freshly prepared 0.001 M of DTNB solution. The changes in yellow chromogen absorbance were analysed by a spectrophotometer device at 412 nm of wavelength. The reference standard was prepared between 0 to 100 μ M of GSH solution.

Estimation of AP Activity: The level of AP activity is indicator of lysosomal & mitochondrial damage and it was estimated as described by Neil and Horner [49] using a commercial colorimetric acid phosphatase assay kit (Abcam Inc., Boston, United States of America). Briefly, 3 mL of substrate solution was added to 0.5 mL of aliquot samples. The substrate solution was prepared freshly by dissolving 1.49 g of ethylene-diamine-tetra acetic acid, 0.84 g of citric acid, and 0.03 g of p-nitro-phenyl phosphate in 100 mL of distilled water. Finally, the solution mixture pH was adjusted to 5.3 and incubated for 5 minutes at 37°C. Then, 0.05 mL of sample was removed from the test tube solution and added to 9.5 mL of 0.085 N of sodium hydroxide. The basic principle of AP activity is readily converting the p-nitro-phenol phosphate (substrate) by hydrolysis action and it generates the yellow

color chromogen i.e., p-nitro-phenol at alkaline pH. The changes in yellow chromogen absorbances were analysed by a spectrophotometer device at 405 nm of wavelength. The reference standard was prepared between 0 to 20 mM of p-nitro-phenol solution.

Estimation of AXA2 Activity: The level of AP activity is an indicator of lysosomal & mitochondrial damage and it was estimated using a commercial AXA2 ELISA (Colorimetric) kit (Abcam Inc., Boston, United States of America). Briefly, 50 μ L of aliquot samples were mixed with 50 μ L of antibody cocktail solution. The well plate was incubated at room temperature for 1 hour followed by all wells being washed with 350 μ L of washing buffer. The washing procedure was repeated three times. Finally, 100 μ L of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 10 minutes followed by added 100 μ L of stop solution. The changes of absorbance were measured at 450 nm wavelength. The reference standard was prepared with 8.8 to 100 ng/ml of AXA2 as described procedure of the ELISA kit.

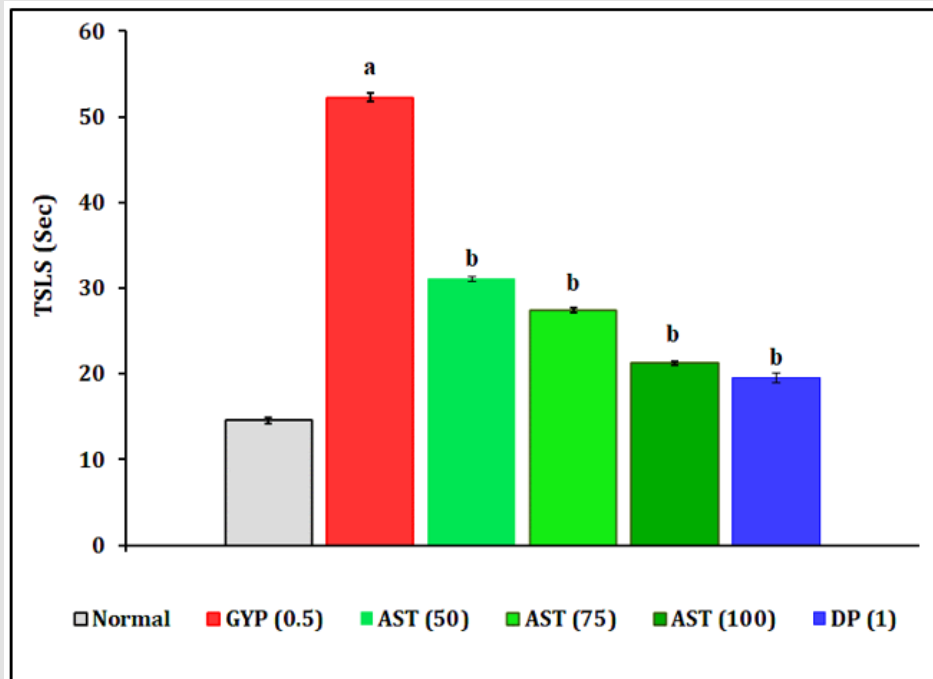
Estimation of Total Protein Level: The level of total protein was estimated as described by Lowry et al. [50]. Briefly, 300 μ L aliquot was combined with 700 μ L of distilled water. Then, 5 ml of Lowry's reagent was added to each test tube sample. These test mixtures were incubated at 37°C for 15 minutes. Thereafter, the 0.5 mL of Folin-Ciocalteu reagents were combined slowly and vortexed for 30 minutes. The changes in purple chromogen absorbances were analysed by a spectrophotometer device at 750 nm wavelength. The reference standard was prepared between 0 to 10 mg of bovine serum albumin solution.

Statistical Analysis: All the results were recorded as mean plus or minus standard deviation (\pm SD). Data secured from all behaviour tests and tissue biomarkers were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test as post-hoc analysis. The GraphPad Prism Version- 5.0 software was used for statistical analysis. A probability value of $p < 0.05$ was considered statistically significant.

Results

Effect of AST in GYP-Induced Horizontal Compartment Response Changes

The exposure of GYP (0.5 mg/L for 30 minutes/day) for 14 consecutive days produced a significant ($p < 0.05$) impairment of neurobehavior pattern in the horizontal compartment response test as an indication of increasing TSLs values when compared to the normal control group. The exposure of AST (50, 75, and 100 mg/L for 30 minutes/day) for 14 consecutive days was shown to neuroprotective action against GYP-induced neurobehavior changes in dose dose-dependent manner. Moreover, the treatment of DP (1 μ g/mL) also showed significant attenuation of GYP-induced neurobehavior changes. The results were illustrated in Figure 1.



Note: Abbreviation: AST, astaxanthin; DP, donepezil; GYP, glyphosate; Sec, seconds; and TSLS, time spent in the lower segment.

Figure 1: Effect of AST in GYP-induced horizontal compartment response changes. Digits in parenthesis indicate the dose of GYP (0.5 mg/L); AST (50 mg/L); AST (75 mg/L); AST (100 mg/L); and DP (1 µg/mL). Data were expressed as mean ± SD, n = 20 zebrafish per group. ^ap < 0.05 Vs normal group. ^bp < 0.05 Vs GYP treated group.

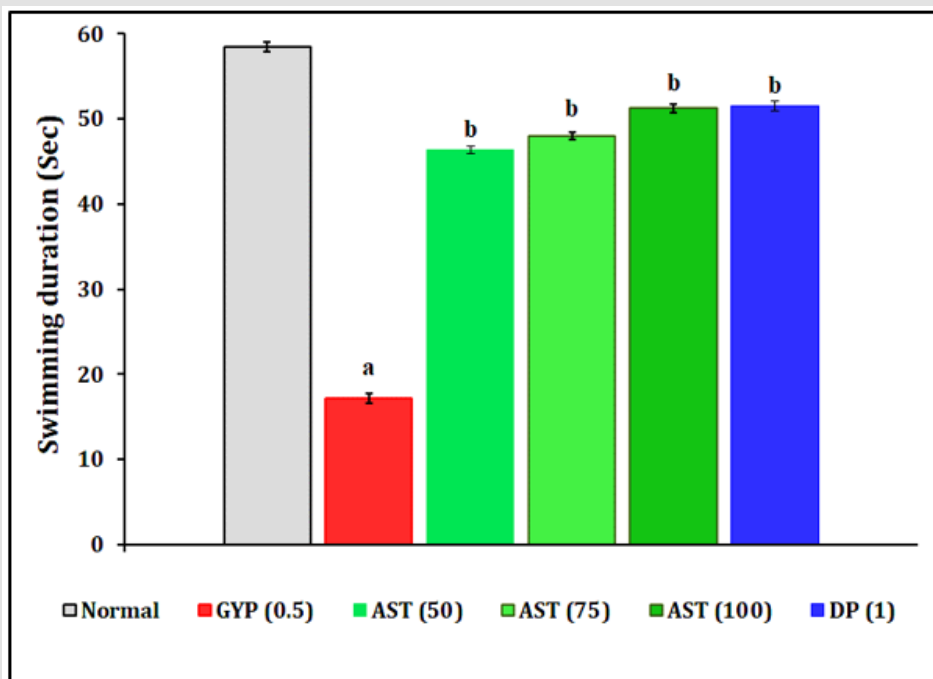


Figure 2: Effect of AST in GYP-induced OMR changes. Digits in parenthesis indicate the dose of GYP (0.5 mg/L); AST (50 mg/L); AST (75 mg/L); AST (100 mg/L); and DP (1 µg/mL). Data were expressed as mean ± SD, n = 20 zebrafish per group. ^ap < 0.05 Vs normal group. ^bp < 0.05 Vs GYP treated group. Abbreviation: AST, astaxanthin; DP, donepezil; GYP, glyphosate; and Sec, seconds.

Effect of AST in GYP-Induced OMR Changes

The exposure of GYP (0.5 mg/L for 30 minutes/day) for 14 consecutive days produced a significant ($p < 0.05$) impairment of visual-motor and neuromuscular functional patterns as an indication of decreasing swimming duration values when compared to the normal control group. The exposure of AST (50, 75, and 100 mg/L for 30 minutes/day) for 14 consecutive days was shown to have neuroprotective action against GYP-induced visual motor and neuromuscular functional changes in dose dose-dependent manner. Moreover, the treatment of DP (1 $\mu\text{g}/\text{mL}$) also showed significant attenuation of GYP-induced visual motor and neuromuscular functional pattern changes. The results were illustrated in Figure 2.

Effect of AST in GYP-Induced SR Changes

The exposure of GYP (0.5 mg/L for 30 minutes/day) for 14 consecutive days produced a significant ($p < 0.05$) impairment of the defensive response pattern as an indication of decreasing percentage (%) SR values when compared to the normal control group. The exposure of AST (50, 75, and 100 mg/L for 30 minutes/day) for 14 consecutive days was shown to have neuroprotective action against GYP-induced % SR changes in dose dose-dependent manner. Moreover, the treatment of DP (1 $\mu\text{g}/\text{mL}$) also showed significant attenuation of GYP-induced % SR changes. The results were illustrated in Figure 3.

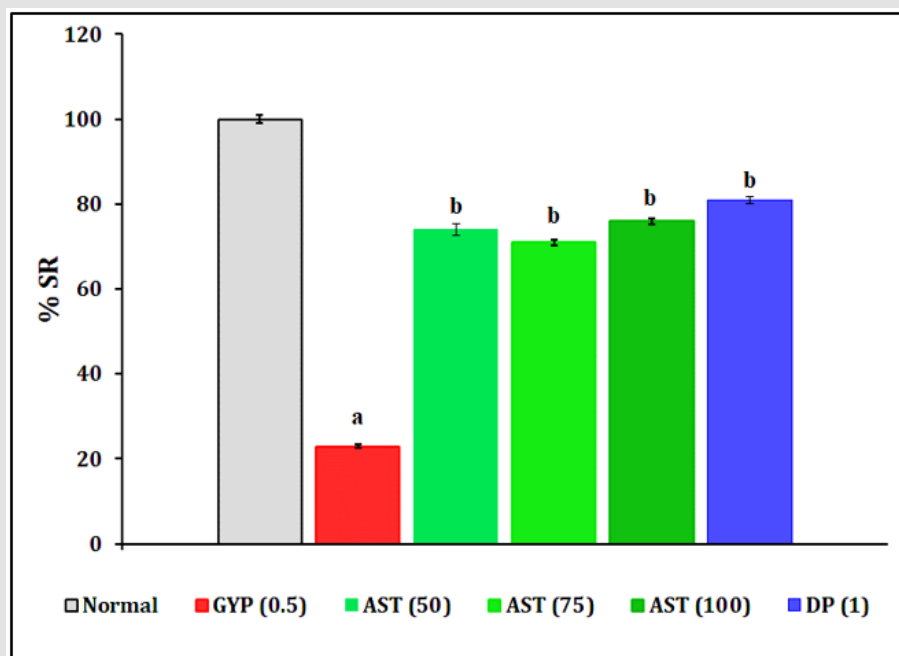
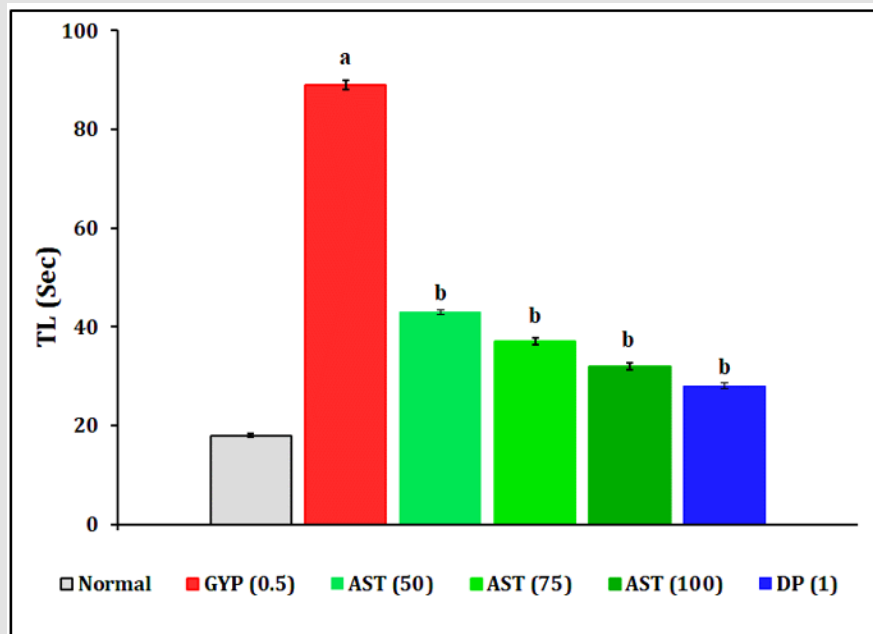


Figure 3: Effect of AST in GYP-induced SR changes. Digits in parenthesis indicate the dose of GYP (0.5 mg/L); AST (50 mg/L); AST (75 mg/L); AST (100 mg/L); and DP (1 $\mu\text{g}/\text{mL}$). Data were expressed as mean \pm SD, $n = 20$ zebrafish per group. ^a $p < 0.05$ Vs normal group. ^b $p < 0.05$ Vs GYP treated group. Abbreviation: AST, astaxanthin; DP, donepezil; GYP, glyphosate; and SR, startle response.

Effect of AST in GYP-Induced T-Maze Test Response Changes

The exposure of GYP (0.5 mg/L for 30 minutes/day) for 14 consecutive days produced a significant ($p < 0.05$) impairment of neuro-cognitive behavior pattern as an indication of increasing TL values when compared to the normal control group. The exposure of AST

(50, 75, and 100 mg/L for 30 minutes/day) for 14 consecutive days was shown to have neuroprotective action against GYP-induced TL values changes in dose dose-dependent manner. Moreover, the treatment of DP (1 $\mu\text{g}/\text{mL}$) also showed significant attenuation of GYP-induced TL value changes. The results were illustrated in Figure 4.



Note: Abbreviation: AST, astaxanthin; DP, donepezil; GYP, glyphosate; Sec, seconds; and TL, transfer latency.

Figure 4: Effect of AST in GYP-induced T-maze test response changes. Digits in parenthesis indicate the dose of GYP (0.5 mg/L); AST (50 mg/L); AST (75 mg/L); AST (100 mg/L); and DP (1 µg/mL). Data were expressed as mean ± SD, n = 20 zebrafish per group. ^ap < 0.05 Vs normal group. ^bp < 0.05 Vs GYP treated group.

Effect of AST on GYP-Induced Tissue Biomarker Changes

The exposure of GYP (0.5 mg/L for 30 minutes/day) for 14 consecutive days produced a significant ($p < 0.05$) rise in the brain AChE activity, & TBARS levels; and reduced the GSH, AP & AXA2 activity levels when compared to the normal control group. The exposure of AST (75 and 100 mg/L for 100 minutes/day) for 14 consecutive days

showed amelioration above tissue biomarkers changes. Whereas the exposure of AST (50 mg/L for 100 minutes/day) for 14 consecutive days did not show any ameliorative effect on the above tissue biomarkers changes. However, the treatment of DP (1 µg/mL) also showed significant attenuation of GYP-induced tissue biomarker changes. The results were tabulated in Table 1.

Table 1: Effect of AST in GYP-induced tissue biomarker changes.

Groups	AChE Activity (µM/mg of protein/min)	GSH (µM/mg of protein)	TBARS (nM/mg of protein)	AP Activity (mM/mg of protein)	AXA2 Activity (ng/mg of protein)
Normal	18.9 ± 1.3	20.6 ± 1.2	2.9 ± 0.4	3.5 ± 0.7	2.9 ± 0.9
GYP (0.5)	63.6 ± 0.9 ^a	4.1 ± 0.7 ^a	19.2 ± 0.7 ^a	1.1 ± 0.9 ^a	0.8 ± 0.5 ^a
AST (50)	56.1 ± 1.1	5.9 ± 1.7	14.6 ± 0.9	1.4 ± 0.4	1.2 ± 0.4
AST (75)	23.2 ± 0.7 ^b	17.6 ± 1.4 ^b	7.2 ± 1.1 ^b	2.3 ± 0.3 ^b	2.1 ± 0.5 ^b
AST (100)	21.1 ± 1.2 ^b	18.5 ± 0.7 ^b	5.7 ± 1.2 ^b	2.8 ± 0.9 ^b	2.9 ± 0.4 ^b
DP (1)	19.0 ± 0.9 ^b	20.1 ± 1.1 ^b	4.2 ± 0.9 ^b	3.2 ± 0.6 ^b	3.1 ± 0.6 ^b

Note: Digits in parenthesis indicate the dose of GYP (0.5 mg/L); AST (50 mg/L); AST (75 mg/L); AST (100 mg/L); and DP (1 µg/mL). Data were expressed as mean ± SD, n = 20 zebrafish per group. ^ap < 0.05 Vs normal group. ^bp < 0.05 Vs GYP treated group. Abbreviation: AChE, acetylcholinesterase activity; AP, acid phosphatase; AST, astaxanthin; AXA2, annexin A2; DP, donepezil; GSH, reduced glutathione; GYP, glyphosate; and TBARS, thio barbituric acid reactive substances.

Discussion

The present study revealed that, the exposure of AST (75 and 100 mg of AST/L) and DP (1 µg/L) significantly ($p < 0.05$) ameliorates the GYP (0.5 mg/L) induced cognitive changes in zebrafish as indicated as decreased TSLs value in three horizontal compartment tests; raising the swimming duration in OMR test; increased the %SR values in startle response test; and reduce the TL values in T-Maze test. In contrast, the exposure to AST (50 mg of AST/L) did not show significant ameliorative effects. These results reveal that AST has potential nootropic actions. Moreover, AST also attenuates the biomarkers (GSH, TBARS, AChE, AP, and AXA2 activities) levels dose-dependent manner similar to the DP exposure group. These results of AST closely mimic the reference drug i.e., DP actions. Marine drugs, AST are potentially altering the neuronal cognitive function due to progressive development of neuronal damage and neurodegenerations via multiple biochemical changes. Acetylcholine is a cholinergic neurotransmitter and it contributes to acute and long-term memory processes of the brain cells [51]. The GYP is a neurotoxic agent and it elevates the levels of AChE levels which leads to cognitive dysfunction [15]. In contrast, low levels of GLY exposure are known to inhibit the activation of AChE actions [52]. AST is known to elevate acetylcholine leads to improved memory functions via the inhibition of AChE activity [33].

The current data also revealed that AST enhances brain memory function via inhibition of GYP-induced AChE activity levels. Experimentally, AST also possesses an antidote-like action against GYP toxicity [53]. Moreover, GYP is known to damage the neuronal tissue with the influence of free radicals and the accumulation of toxic proteins like metallothionein I and II gene expression, inflammation proteins, and mitochondrial apoptotic proteins [54-56]. Similar pathology is established in the progression of neurodegenerative disorders like Alzheimer's disease and other dementia conditions [57]. Moreover, GYP potentially alters broad transcriptomic responses especially AXA2 levels [58]. AST also regulates the mitochondrial function via the reduction of free radicals by GSH and prevents lipid peroxidation products (TBARS) leading to attenuating the neurodegenerations [23,59]. Moreover, the AST attenuates the pyrethroid insecticide like lambda-cyhalothrin-induced neuronal damage [60], and broad-spectrum contact herbicide i.e., flumioxazin-induced cellular damage in zebrafish embryos [61]. AP is a lysosomal and mitochondrial marker for cell damage. The reduction of AP biosynthesis in neuronal cells potentially causes neurodegeneration via aggregation of α -synuclein proteins and lysosomal destructions [62,63]. Similarly, the destruction of AXA2 activity causes neuronal death and neurodegeneration via a lack of p53 protein functions [30]. The exogenous recombinant AXA2 therapy prevents traumatic brain injury-induced neurological dysfunctions via the regulation of AXA2 deficiency [31,32].

Recent evidence also revealed that AST enhances hippocampal neuronal sprouting and improves spatial memory functions via GABAergic interneuron actions [64,65]. GLY causes the potential suppression

of acid and alkaline phosphatase activity [66]. Here, AST acts as a detoxification enzyme in neurodegenerative disorders via the regulation of neurovascular functions. Zebrafish are widely accepted animal species of lower vertebrates for the evaluation of neurocognitive functions due to genetic similarities of the human genome [67]. The limitations of this study are very narrow; this research work covered the evidence of neurocognitive functions with behavioral and biochemical assessment. However, the assessment of AP and AXA2 expression levels may provide additional evidence for the neurodegenerative and neuroprotective actions of AST. Based on current results, this work was taken into account to extend the study to explore this neuroprotective action of AST against the GYP in higher vertebrate animals with assessment of AP and AXA2 expression and immune-histopathological observations.

Conclusion

The AST attenuates the GYP-induced cognitive dysfunction viz reduction of free radical generations, lipid peroxidation, and regulation of cholinergic neuronal functions, AP, and AXA2 actions. Hence, it may be a useful novel nootropic agent for phosphonate type of herbicide-associated neurotoxicity and cognitive dysfunctions. Moreover, extensive studies are needed to establish complete therapeutic potential and a better understanding of pathophysiological processes in various conditions. Our research team has taken the initiative to extend this study to overcome the limitations such studies are under investigation.

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Conflict of Interests

Authors declare that there is no conflict of interests.

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