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Original article

Isatis phytogenic relieved atrazine induced growth retardation, hepato-renal dysfunction, and oxidative stress in Nile tilapia

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ABSTRACT

The influence of herbicides causes health and economic loss, which requires innovative solutions to sustain the aquaculture industry. In this regard, dietary isatis is included in Nile tilapia diets to relieve atrazine (ATZ)-induced growth retardation, hepato-renal dysfunction, and oxidative stress. The first and second groups offered the control diet (control), while the third and fourth groups offered the isatis supplemented diet (1%). Meantime, half of the water was replaced and mixed with ATZ (1.39 mg/L) in the second and fourth groups for 30 days. The group of fish delivered isatis had significantly enhanced FBW, WG, and SGR, while fish intoxicated with ATZ had meaningfully impaired growth behavior ($p < 0.05$). Further, the FCR was improved by isatis, and ATZ resulted in the worst FCR among the groups. Interestingly fish fed isatis and exposed with ATZ (88.89%) had a higher survival rate than fish exposed with ATZ without isatis feeding, and both are lower than the control (97.78%) ($p < 0.05$). The histological structure in the isatis-treated groups showed distinguished enhancement and branching of the intestinal villi. The intestine of ATZ–treated fish revealed damage and inflammatory cell infiltration in the intestinal mucosa with separation of lining epithelium. Generally, fish fed isatis and intoxicated with ATZ had lower uric acid, urea, creatinine, ALT, and AST and higher total protein, globulin, and albumin than fish exposed with ATZ without feeding with isatis ($p < 0.05$). Markedly, fish-fed isatis had the highest SOD, CAT, GPx, and the lowest MDA level compared to the other groups ($p < 0.05$). Meanwhile, fish exposed with ATZ had the worst SOD, CAT, GPx, and the highest MDA level compared to the other groups ($p < 0.05$). In summary, dietary isatis relieved ATZ induced growth retardation, hepato-renal dysfunction, and oxidative stress in Nile tilapia.
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1. Introduction

Atrazine is a widespread herbicide that has been licensed for the agriculture sector since 1958 in the USA for weeds growth inhibition in sugarcane, sorghum, and corn crops (Solomon et al., 1996). Atrazine has a long life and can stay active for greater than 200 days in the drainage agriculture water (de Albuquerque et al., 2020). Many countries banned atrazine usage, but still, other counties massively apply atrazine in the agriculture sector (Khatoon, Rai, 2020). Atrazine derivatives contaminate the groundwater, rivers, lakes, and fishponds and induce chronic ecotoxic impacts on biotic and abiotic

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organisms (Singh et al., 2018). Chronic exposure with atrazine upraised the inflammation, oxidative stress, and infection with pathogens in finfish species (Blahova et al., 2020; Owolabi, Omotosho, 2017; Toughan et al., 2018). Nile tilapia (*Oreochromis niloticus*) is cultured worldwide and challenges atrazine exposure in the fishponds (Oliveira et al., 2018). Nile tilapia contributes to food chain sustainability due to its high productivity and commercial value (Dawood et al., 2021b; Van Doan et al., 2019). The chronic water intoxication with atrazine caused immunotoxic, genotoxic, and oxidative stress alterations leading to a reduction in the growth rate and feed efficiency of Nile tilapia (Abdel-Warith et al., 2021; Neamat-Allah et al., 2020). Concurrently fish becomes more susceptible to infection, which threatens the feasibility of aquaculture activity in many agricultural-based economics.

Natural immunostimulants are appropriate for aquaculture to minimize chemotherapies' application (Dawood, 2021; Lieke et al., 2020). Indeed, phytogenic derived substances are approved as effective, friendly alternatives in aquaculture (Sinha et al., 2021; Vazirzadeh et al., 2020; Zhu, 2020). Isatis root powder is extracted initially from *Isatis indigotica* (Cruciferae) and cultured in several Asian, middle east, and European countries (Speranza et al., 2020). Isatis extract has several functional components such as glycoproteins, polysaccharides, carotenoids, essential oils, and phenols (Taviano et al., 2018). Markedly, isatis resulted in antibacterial, antiviral, antioxidative, anti-inflammation, anti-cancer, and immunomodulation effects in vivo and in vitro (Han et al., 2011; Kong et al., 2004). The incorporation of isatis in the diets of *Carassius auratus* gibelio (Xiao, 2003), Jian common carp (*Cyprinus carpio* var. Jian) (Jichang, Zaohe, 2002), and pufferfish (*Takifugu obscurus*) (Song et al., 2018) resulted in improved production, immune, and antioxidative responses. However, no studies investigated the effect of isatis on Nile tilapia performances.

The study aimed at exploring the impact of waterborne atrazine-induced hepato-renal failure and oxidative stress in Nile tilapia and the protective role of dietary isatis extract.

2. Materials and methods

2.1. Fish and feed

Two sets of experimental diets were prepared by mixing all ingredients (Supplementary file). The control diet contains the basal formulation without including isatis, while the second diet was prepared by mixing isatis (Free Trade Company, Albeheira, Egypt) at 1 % by following Xiao (2003). The control and isatis supplemented diets were well mixed, and water and fish oil were included; then, diets were pelleted with a laboratory pelleting machine to have 2–3 mm die pellets. The pellets were kept drying in the oven at 45–50 °C then stocked in plastic bags at 4 °C till using in the study. Nile tilapia were obtained from a local farm in the Baltim area and transported to the Fish Nutrition Laboratory, Baltim Unit, National Institute of Oceanography and Fisheries. Fish were adapted to the laboratory conditions for ten days before beginning the trial in 1000 L concrete tanks fixed with a flow-through system. During the adaptation period, fish fed the basal diet twice daily (08:00 and 15:00). Then fish was checked for the initial weight (16.77 ± 0.10 g), and 15 fish were stocked in 12 glass aquaria (70 L). Each group was represented with three aquaria, and each aquarium was fixed with continuous aeration while the water was replaced daily with fresh dechlorinated water.

2.2. Experimental design

Four groups were assigned in this study, where each group contains three glass aquaria (15 fish/ each aquarium). The first and second groups offered the control diet (control), while the third and fourth groups offered isatis supplemented diet. Half of the water used in control and isatis groups was daily exchanged with dechlorinated freshwater. Half of the water was replaced and mixed with atrazine (ATZ) daily in the second and fourth groups. The ATZ (98% purity; Sigma-Aldrich Company, St. Louis, USA) dose of toxicity was 1/5 96-hr LC_{50} (1.39 mg/L) based on the findings of Neamat-Allah et al. (2020). The trial lasted for 30 days, and fish fed the respective diets twice daily (08:00 and 15:00). The water quality indices were kept at 25.22 ± 0.24 °C, 7.11 ± 0.4, 6.12 ± 0.41 mg/L, and 0.2 ± 0.01 mg/L for temperature, pH, dissolved oxygen, and total ammonia during the trial.

2.3. Final sampling

All fish were starved 24 h before the final sampling. Then fish were weighed individually (FBW, g) and counted to calculate the weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate.

$$WG = 100 \times (FBW - \text{initial weight (IBW, g)})/IW\ (g)$$

$$SGR\ (\text{percent/day}) = 100 \times (\ln FBW\ (g) - \ln IBW\ (g))/\text{days}$$

$$FCR = \text{total dry feed intake (FI, g)}/\ (FBW\ (g) - IBW\ (g))$$

$$\text{Survival}\ (\%) = 100 \times \text{final fish number/initial fish number}$$

Then fish were anesthetized with 100 mg/L tricaine methanesulfonate, and blood was collected from 3 fish/aquarium using 5 mL gauge syringes from the caudal vein. Half of the blood was kept in EDTA-heparinized tubes and immediately used for hematological analysis. The remaining blood was kept in non-heparinized tubes for serum collection. After 2 h, blood samples were centrifuged at 3000 rpm/ 15 min at 4 °C; then, serum was separated and kept at − 20 °C for further analysis. Besides, three fish per aquarium were dissected, and their intestines were extracted to analyze the histological features, while livers were dissected for measuring the antioxidative capacity. The homogenates of livers were prepared by rinsing livers in ice-cold Phosphate-Buffered Saline (PBS) (pH 7.5; 1 g per 10 mL). It was then homogenized and centrifuged at 8000 rpm for 5 min, and the supernatant was collected and stored at 4 °C for further analysis.

2.4. Blood analysis

Serum total proteins and albumins were determined, according to Doumas et al. (1981) and Dumas (1972), while globulins content was calculated mathematically. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, urea, and uric acid were detected by RA-50 chemistry analyzer (Bayer) using readymade chemicals (kits) supplied by Spinreact Co. Spain, following the manufacturer's instructions.

Serum and liver homogenate malondialdehyde (MDA) levels were determined by using the thiobarbituric acid method and were calorimetrically clarified using a commercial kit (LPO, OXIS Int., USA) as described by Ohkawa et al. (1979). The serum and liver homogenate samples were used to detect superoxide dismutase (SOD) (McCord, Fridovich, 1969), catalase (CAT) (Aebi, 1984), and glutathione peroxidase (GPx) (Habig et al., 1974) activities using colorimetric methods by commercial fish-specific kits (Biodiagnostic Co., Giza, Egypt).

2.5. Histomorphology

The dissected intestine samples were cut into pieces of approximately 0.5 cm^3 and fixed in neutral buffered formaldehyde 10% solution for 24 h (Gewaily, Abumandour, 2021). The samples were then dehydrated in ascending grades of alcohol, cleared with xylene, and embedded in paraffin wax. Then five μm thick sections were cut using Leica rotatory microtome (RM 20352035; Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin and eosin. Finally, the tissue sections were examined using a BX50/BXFLA microscope (Olympus, Tokyo, Japan).

2.6. Statistical analysis

Shapiro-Wilk and Levene tests confirmed normal distribution and homogeneity of variance. The obtained data were subjected to one-way ANOVA. Differences between means were tested at $p < 0.05$ level using the Duncan test as a post-doc test. All the statistical analyses were done via SPSS version 22 (SPSS Inc., IL, USA).

3. Results

3.1. Growth performance

The results of growth traits were markedly influenced by ATZ toxicity and isatis feeding (Table 1). The group of fish delivered isatis had significantly enhanced FBW, WG, and SGR, while fish intoxicated with ATZ had meaningfully impaired growth behavior ($p < 0.05$). Further, the FCR was enhanced by isatis, and ATZ resulted in the worst FCR among the groups (Table 1). Fish fed the isatis and exposed to ATZ had similar growth behavior and FCR with fish fed the control diet without ATZ toxicity ($p > 0.05$). Fish-fed isatis had the best survival rate (100%), but fish exposed to ATZ had the worst survival (80%) with marked significant differences ($p < 0.05$) (Table 1). Interestingly fish fed isatis and exposed with ATZ (88.89%) had a higher survival rate than fish exposed with ATZ without isatis feeding, and both are lower than the control (97.78%) ($p < 0.05$).

3.2. Intestinal histology

The morphological structure of the intestine of Nile tilapia in the control fish revealed normal, intact intestinal villi, lamina propria submucosa, tunica muscularis, and tunica serosa throughout the three parts of the intestine: duodenum, jejunum, and ileum (Fig. 1. A1, M1, P1 respectively). The histological structure in the isatis-treated groups (Fig. 1. A3, M3, P3) showed distinguished enhancement and branching of the intestinal villi. The intestine of ATZ–treated fish revealed damage and inflammatory cell infiltration in the intestinal mucosa with separation of lining epithelium (Fig. 1. A2, M2, P2). The ATZ/isatis-subjected group showed detectible improvement of the intestinal villi in addition to immune cells infiltration near the base of the intestinal villi (Fig. 1. A4, M4, P4).

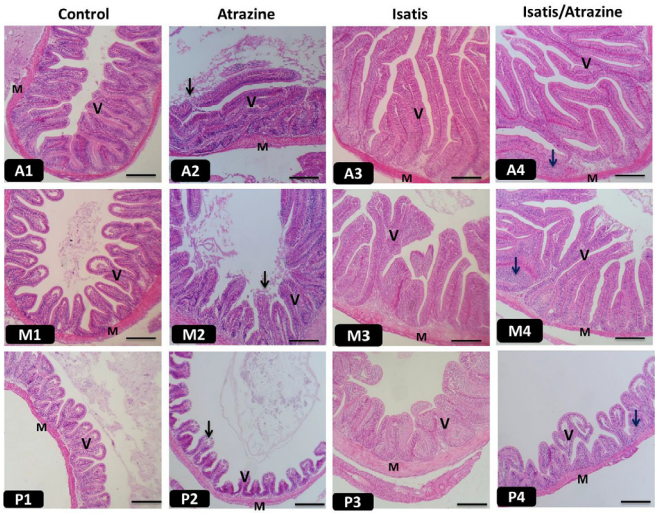

Fig. 1. Histomicrograph of the of the intestine of the Nile tilapia including duodenum (upper panel; A1-A4), jejunum (middle panel; M1-M4) and ileum (lower panel; P1-P4) in the control group as well as other treated (Atrazine, Isatis, and Isatis/Atrazine) groups. The histological structure in the control group as well as the Isatis-treated groups showed normal, intact intestinal villi (V), lamina propria sub mucosa, tunica muscularis (M) and tunica serosa with improved growth of the intestinal villi in the Isatis-treated group. The intestine of atrazine–treated fish revealed damage and inflammatory cell infiltration in the intestinal mucosa with separation of lining epithelium (black arrow). The Atrazine/Isatis-subjected group showed obvious improvement of the intestinal villi in addition to immune cells infiltration near the base of the intestinal villi (dark blue arrow). Stain H&E. Bar: 100 μm.

3.3. Blood biochemical indices

Generally, fish fed isatis and intoxicated with ATZ had lower uric acid, urea, creatinine, ALT, and AST and higher total protein, globulin, and albumin than fish exposed with ATZ without feeding with isatis ($p < 0.05$) (Fig. 2). The serum uric acid, urea, creatinine, ALT, and AST are markedly increased by ATZ exposure and reduced by isatis feeding ($p < 0.05$). On the other hand, the serum total protein, globulin, and albumin are markedly increased by ATZ exposure and reduced by isatis feeding ($p < 0.05$). Fish in the control group had higher uric acid levels than fish delivered isatis and a lower uric acid level than fish exposed with ATZ with or without isatis feeding ($p < 0.05$) (Fig. 2A). Besides, fish in the control group had higher ($p < 0.05$) urea (Fig. 2B), AST (Fig. 2D), and ALT (Fig. 2E) than fish-fed isatis and similar levels ($p > 0.05$) with fish-fed isatis and exposed with ATZ. However, fish in control had higher ($p < 0.05$) creatinine levels than fish-fed isatis with or without ATZ exposure (Fig. 2C). Fish in the control group had lower ($p < 0.05$) total protein (Fig. 2F), globulin (Fig. 2G), and albumin

Table 1
Growth performance of Nile tilapia fed isatis and exposed with atrazine.

	Control	ATZ	Isatis	Isatis/ATZ
IBW (g)	16.38 ± 0.24	16.28 ± 0.02	16.44 ± 0.07	16.31 ± 0.16
FBW (g)	33.90 ± 0.29^{b}	27.90 ± 1.47^{c}	39.67 ± 0.80^{a}	33.95 ± 0.32^{b}
WG (%)	106.96 ± 1.97^{b}	71.39 ± 8.77^{c}	141.35 ± 5.23^{a}	108.13 ± 0.20^{b}
SGR (%/day)	2.42 ± 0.03^{b}	1.79 ± 0.17^{c}	2.94 ± 0.07^{a}	2.44 ± 0.00^{b}
FCR	1.59 ± 0.03^{b}	2.61 ± 0.09^{a}	1.31 ± 0.06^{c}	1.57 ± 0.05^{b}
Survival (%)	97.78 ± 2.22^{b}	80.00 ± 3.85^{d}	100.00 ± 0.00^{a}	88.89 ± 2.22^{c}

Means ± S.E. in the same row with different letters, differ significantly ($p < 0.05$).

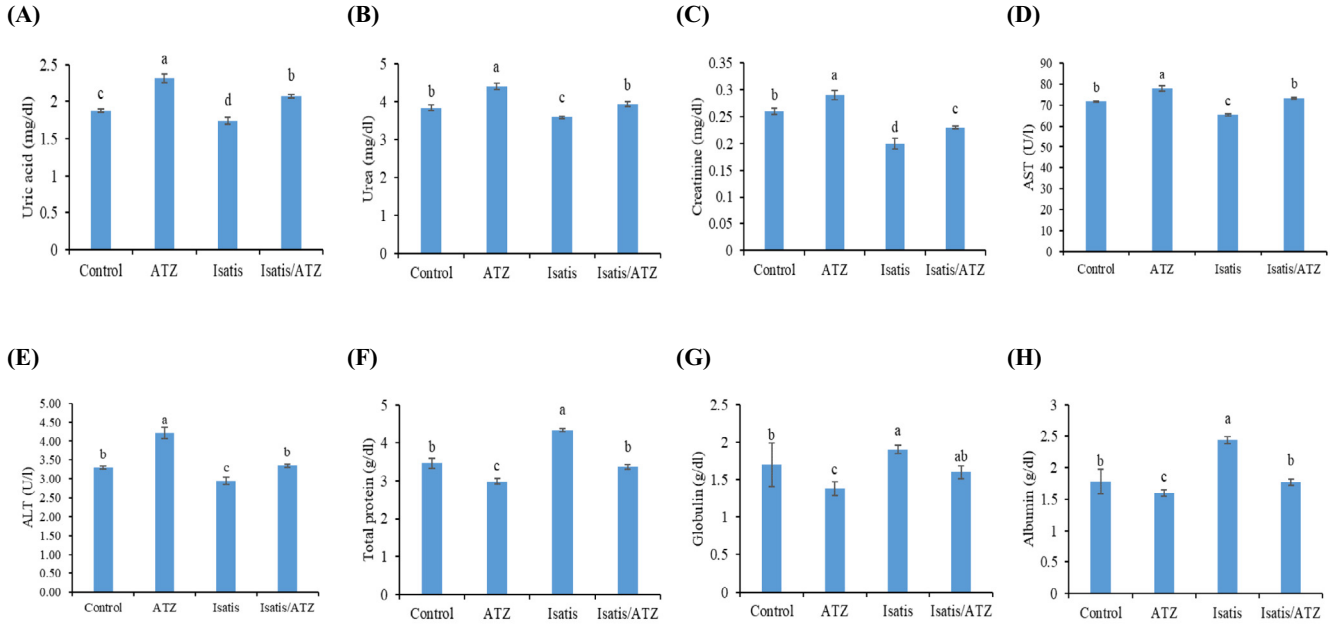

Fig. 2. The blood biochemical traits of Nile tilapia fed isatis and exposed with atrazine. Bars present means ± S.E. with different letters, differ significantly ($p < 0.05$) (n = 3). The groups are the control, fish fed dietary isatis, fish fed basal diet and exposed with atrazine (ATZ), and fish fed isatis and exposed with ATZ (isatis/ATZ).

(Fig. 2H) than fish-fed isatis and similar levels ($p > 0.05$) with fish-fed isatis and exposed with ATZ.

3.4. Antioxidative capacity

The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and malondialdehyde level (MDA) were detected in the serum and liver samples of Nile tilapia under the current trial conditions (Fig. 3). Markedly, fish-fed isatis had the highest SOD (Fig. 3A), CAT (Fig. 3B), GPx (Fig. 3C), and the lowest MDA level (Fig. 3D) compared to the other groups ($p < 0.05$). Meanwhile, fish exposed to ATZ had the worst SOD (Fig. 3A), CAT (Fig. 3B), GPx (Fig. 3C), and the highest MDA level (Fig. 3D) compared to the other groups ($p < 0.05$). Interestingly, fish fed isatis and exposed with ATZ had similar ($p > 0.05$) SOD, CAT, and GPx compared with fish in the control group. The MDA level in serum was similar ($p > 0.05$) in fish of control and isatis/ATZ groups. However, fish fed isatis and exposed to ATZ had lower MDA levels in the liver than the control fish ($p < 0.05$).

4. Discussion

The main harmful impact of atrazine is the generation of free radicals (ROS) in the organism's entire body, ending with severe oxidative stress (Chen et al., 2015). The waterborne herbicides attack the gills and skin of fish and cause inflammatory features resulting from oxidative stress (Paulino et al., 2012a; Sula et al., 2020; Vajargah et al., 2021). Besides, atrazine contamination induces lipid peroxidation and cell damage, which disrupts fish's respiration function and osmoregulation (Abdel-Warith et al., 2021; Oliveira et al., 2018). Accordingly, all physiological, metabolic, and biochemical functions deteriorate, leading to growth reduction and immunosuppression (Chiste et al., 2021). Therefore, using natural antioxidative pharmaceutics is recommended to relieve atrazine herbicides' toxic impacts on finfish species. Isatis contains enough active phytochemical substances involved in regulating fish's immune and antioxidative responses (Karakoca et al., 2013; Speranza et al., 2020).

The growth rate of Nile tilapia-fed dietary isatis is meaningfully enhanced compared with the control and atrazine groups. However, fish intoxicated with atrazine without feeding isatis displayed a reduced growth rate with high mortality level. The impaired growth and feed performances are directly affected by atrazine toxicity, which induces retardation in metabolic and physiological functions (Song et al., 2018). The results also showed inflammatory features in the intestinal sections responsible for digesting and absorbing the feed nutrients. In the same line, Abdel-Warith et al. (2021), Gewaily et al. (2021), and Dawood et al. (2020) reported that Nile tilapia exposed to pesticides, herbicides, and insecticides had abnormal intestinal features and attributed these results to inflammation induced by oxidative stress and lipid peroxidation. On the other hand, the improved growth rate of Nile tilapia-fed dietary isatis is probably attributed to the antibacterial and anti-inflammatory potential in reducing the harmful microorganisms in the intestine and regulating intestinal immunity (Speranza et al., 2020). Behind, isatis contains enough polysaccharides involved in improving the functionality of beneficial microorganisms to act as digestive factors (Song et al., 2018). In this regard, Nile tilapia received isatis showed enhanced feed utilization (FCR) coincided with regulated intestinal features with high digestion capacity. In parallel, Song et al. (2018) stated that pufferfish fed dietary isatis had enhanced growth performance and feed efficiency.

As long as fish's health status is satisfactory, there should be no high levels of mortalities that generally occur due to high stress and lowered immunity (Dawood, 2021). The results showed a high survival rate in groups of Nile tilapia-fed isatis but low survival in groups intoxicated with atrazine. These results confirm that tilapia delivered isatis had a health condition while fish exposed to atrazine suffer from impaired physiological and metabolic functions.

High oxidative stress damages the entire body cells and impairs their functions (Hodkovicova et al., 2020; Jin et al., 2010). Toxicity with atrazine is known to produce lipid peroxides involved in impairing tissue functions (Paulino et al., 2012b). Liver tissue is responsible for detoxifying toxins and regulating metabolic functions and can be evaluated by measuring ALT and AST enzyme levels (Banaee et al., 2013). It has been reported that pesticides,

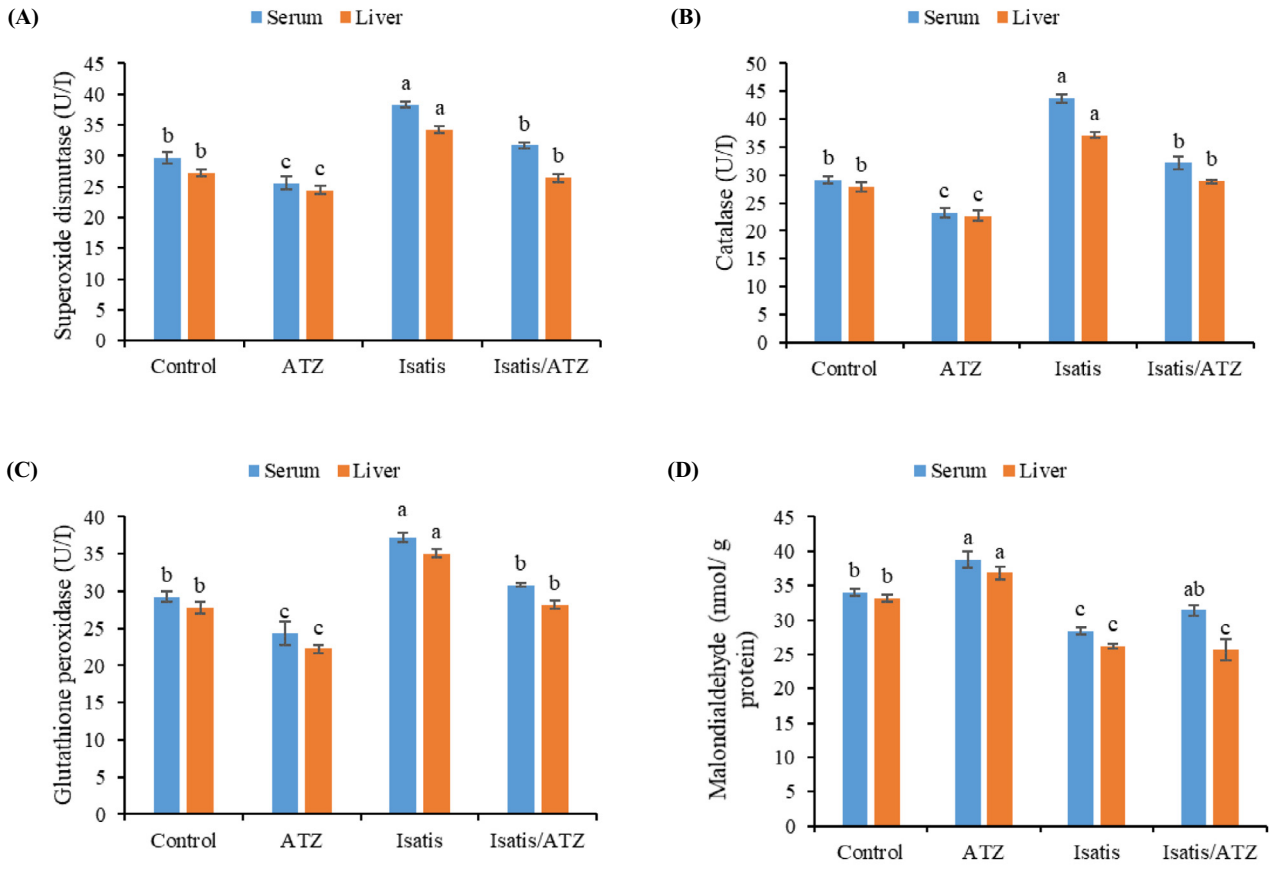

Fig. 3. The antioxidative status of Nile tilapia fed isatis and exposed with atrazine. Bars present means ± S.E. with different letters, differ significantly ($p < 0.05$) (n = 3). The groups are the control, fish fed dietary isatis, fish fed basal diet and exposed with atrazine (ATZ), and fish fed isatis and exposed with ATZ (isatis/ATZ).

herbicides, and insecticides can damage hepatocyte cells and cause high secretion of ALT and AST enzymes in the bloodstream, thereby impaired liver function (Blahova et al., 2014). Interestingly, fish treated with isatis had regulated ALT and AST, and fish treated with both isatis and atrazine had similar levels of ALT and AST when compared with the control. The results suggest that dietary isatis is required in tilapia diets to protect the liver from atrazine-induced oxidative stress.

The blood albumin transfer hormones, enzymes, and digested nutrients (Quinlan et al., 2005), while globulin is involved in the entire body's immune response, and both are produced by the liver tissue (Meyer et al., 2016). The total amounts of albumin and globulin are known as the blood total protein, which increases in high metabolic and immunity status and lowers by stress, toxicity, and infection in the fish body. This study showed high albumin, globulin, and total protein in the blood of Nile tilapia received isatis. However, low levels were shown in fish exposed to atrazine which, in line with previous studies, concluded reduced albumin, globulin, and total protein levels by pesticide toxicity (Abdel-Warith et al., 2021; Abdo et al., 2021; Dawood et al., 2020).

Protein's metabolism ends with urea production, which is filtered by the kidney and secreted outside the body (Al-Daghri et al., 2017). Besides, the kidney is responsible for releasing creatinine and uric acid produced in high amounts in case of kidney failure (Xia et al., 2016). The results showed high urea, uric acid, and creatinine levels in tilapia exposed with atrazine and low levels in fish treated with isatis. Atrazine is one of the toxins involved in impairing the antioxidative status leading to kidney failure and disruption of urea, uric acid, and creatinine filtration (Khoshnood et al., 2015; Stara et al., 2020). However, low levels of urea, uric acid, and creatinine in the blood of fish-fed isatis indicate the health status of fish.

Toxicity with herbicides causes severe stress involved in ROS production, leading to oxidative stress and lipid peroxidation (Chen et al., 2015; Paulino et al., 2012b). In chronic toxicity cases, the high ROS levels develop antioxidative enzyme production (SOD, CAT, and GPx) to cope with the impacts of oxidative stress (Chen et al., 2015). The accumulation of free radicals and lipid peroxides led to inflammation and cell function loss (Blahová et al., 2013; Xing et al., 2012). The gills, kidneys, intestine, and liver organs not far from oxidative stress resulted from herbicides toxicity. Chronic oxidative stress causes dysfunction of gills, kidneys, intestine, and liver, therefore, unstable respiration, detoxification, and osmoregulation, leading to impaired metabolic function, immune and antioxidative responses (Dawood et al., 2021a; de Albuquerque et al., 2020). Besides, the local intestinal digestion and immune capacity can deteriorate in case of high oxidative stress (Dawood, 2021). Markedly the results showed impaired antioxidative capacity in tilapia exposed to atrazine; however, dietary isatis caused upregulated antioxidative capacity. In the same sense, pufferfish fed dietary isatis showed improved antioxidative capacity (Song et al., 2018). Isatis is rich in polyphenols and is known as natural antioxidants to scavenge free radicals since phenolic groups are excellent nucleophiles and can inhibit lipid peroxidation (known as MDA) via binding with free radicals (Speranza et al., 2020).

5. Conclusion

In conclusion, dietary isatis can be approved as a natural antioxidant and antibacterial source for Nile tilapia wellbeing. Dietary isatis relieved the toxic impacts of atrazine exposure on the growth behavior, hepato-renal function, and antioxidative capacity of Nile tilapia. Further studies are required to understand the possible potential role of isatis to ameliorate waterborne toxicants' impacts in finfish species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.08.072.

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