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Dietary garden cress (*Lepidium sativum*) seeds mitigate the effect of aflatoxin B1 contamination on growth, antioxidant status, AFB1 residues, immune response, and tissue architecture of *Oreochromis niloticus*

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ABSTRACT

The contamination of fish diets with aflatoxin B1 (AFB1) poses a significant hazard to the aquaculture sector. This study investigates the efficacy of supplementing Nile tilapia (*Oreochromis niloticus*) diets with garden cress (*Lepidium sativum*) seeds (GCS) to mitigate the adverse effects of AFB1. For this purpose, a factorial design was utilized, consisting of a 2×3 arrangement of treatments. This included two contamination levels of AFB1: (0 and 2 mg kg^{-1} diet). Within each contamination level, three dietary levels of GCS were used (0 %, 1 %, and 2 %). The study involved 270 healthy *O. niloticus* with an average weight of 25.14 ± 0.39 g (45 fish/group and 15 fish/replicate). The duration of the trial was 60 days. The study evaluated growth performance, hematological indices, hepatorenal function, immune and antioxidant indicators, histomorphology of organs, AFB1 residues in muscles, and fish resistance to *Aeromonas hydrophila*. The results indicated that GCS dietary supplementation significantly counteracted AFB1-induced growth retardation and reduced fish survival. Additionally, GCS effectively reversed the anemia, leukopenia, hypoproteinemia, hypoalbuminemia, and hypoglobulinemia that were produced by AFB1. Moreover, GCS dietary supplementation significantly suppressed the AFB1-induced increase in serum bilirubin, hepatic enzymes, kidney damage products, cortisol, and glucose. Additionally, the inclusion of GCS in the fish's diet effectively eliminated the accumulation of AFB1 in their muscles. Moreover, GCS supplementation demonstrated significant restoration of depleted antioxidant and innate immune components induced by AFB1. Furthermore, GCS significantly reduced the degenerative changes in renal, hepatic, splenic, and small intestine tissues caused by AFB1. Fish fed a GCS-fortified diet and exposed to AFB1 displayed restored disease resistance when challenged with *A. hydrophila*. In conclusion, supplementing Nile tilapia diets with GCS at 2 %, even in the presence the AFB1 contamination, significantly enhanced the fish growth, improved health, and increased disease resistance while reducing AFB1 residues, thereby benefiting consumer health.

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1. Introduction

Aflatoxins are a small-molecular weight compounds produced by *Aspergillus parasiticus* and *Aspergillus flavus* fungi. They are commonly found in humid and hot regions and can contaminate crops before or after harvesting, particularly due to improper storage methods (Anater et al., 2016). Aflatoxin B1 (AFB1) is one of the main feed-borne toxins that poses challenges to aquaculture development, particularly with the increasing use of plant-based component in fish feeds (Hussain et al., 2017). AFB1 contamination has been detected in over 50 % of fish feeds in several countries, raising concerns for food safety (Barbosa et al., 2013; Fallah et al., 2014; Deng et al., 2010). No established upper limit is designated explicitly for AFB1 content in aquafeed raw materials (Namulawa et al., 2020). However, authorities such as the World Health Organization (WHO), the US Food and Drug Authority (FDA), and the East African Community (EAC) provide guidelines on permissible limits of AFB1 in food and feeds (WHO: 5 μg/kg, FDA: 20 μg/kg, EAC: 10 μg/kg) (Mutiga et al., 2014).

Numerous adverse effects of AFB1 on fish have been documented, with the liver and kidney being the primary targets (Hussain et al., 2017; Nunes et al., 2019). Liver damage and metabolic abnormalities have been attributed to AFB1 in fish (Naiel et al., 2019; Zeng et al., 2019). Besides, AFB1 accumulation in fish liver and muscle raises concerns for food safety throughout the food chain (Huang et al., 2019).) Studies have shown that AFB1 induces the generation of reactive oxygen species and BAX protein expression in fish, leading to tissue damage, toxic responses, and impaired growth and health (Dey and Kang, 2020; Zeng et al., 2019; Marin and Taranu, 2012).

Clay and mineral adsorbents have been used to mitigate AFB1 contamination in diets (Alinezhad et al., 2017; Hussain et al., 2017), but recent research has highlighted their potential hazards to fish health (Elliott et al., 2020). As a result, a growing interest is directed toward using eco-friendly, safe, and natural alternatives as aquafeed supplements to combat AFB1 contamination (Abdel-Daim et al., 2020; Naiel et al., 2019). Garden cress (GC, *Lepidium sativum* L.) is a rapidly growing annual plant originally native to Egypt and western Asia but is now cultivated in worldwide (Singh and Paswan, 2017). The GC seeds (GCS) are rich in protein, iron, calcium, alpha-linolenic acid, and various vitamins such as C, A, B2, B9, and K (Prajapati and Dave, 2018). They also contain important phytochemical constituents such as flavonoids, triterpene, glucosinolates, alkaloids, saponins, sterols, and tannins (Behrouzian et al., 2014). Additionally, GCS have demonstrated beneficial biological properties, including antioxidant and anti-inflammatory activities (Abu-Khudir et al., 2023).

Few studies have explored the possible beneficial role of GCS as an aquafeed supplement. In the latest study, Bilen et al. (2018) confirmed that adding an ethanolic extract of the whole GC plant (1 and 2 g/kg feed) to juvenile common carp (*Cyprinus carpio*) diets enhanced their growth, immune responses, digestive enzyme activities, and resistance to *Edwardsiella tarda* and *Aeromonas hydrophila*. Yet, a gap still demands more research on the efficacy of using GCS in freshwater fish diets in case of contamination like AFB1. Nile tilapia (*Oreochromis niloticus*) is predominantly farmed in tropical and subtropical regions where AFB1 contamination may be prevalent, and studies have shown negative impacts on various physiological parameters in tilapia even with low levels of AFB1 (Zychowski et al., 2013). Hence, in the current study, a 60-day feeding experiment was conducted to assess the effectiveness of GCS dietary supplements in mitigating the adverse impacts of AFB1 dietary contamination in Nile tilapia, *Oreochromis niloticus*.

2. Material and methods

2.1. Preparation and gas chromatography-mass spectrometry (GC-MS) characterization of GCS

The GCS were acquired in dry packets from a local market in Zagazig, Egypt. Botanical identification and verification of the seeds were examined and confirmed by a botanist in the Department of Plants, Faculty of Science, Zagazig University, Zagazig, Egypt. The GCS were subsequently rinsed, dried, and crushed by an electrical blender. The crushed GCS were then stored at 4 °C until further use. The active compounds of the powdered GCS was determined using a Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) equipped with a direct capillary column TG-5MS (0.25 mm × 30 m × 0.25 μm). The analysis followed the protocol described by Abu-Rumman (2018). The column oven temperature was initially set at 50 °C for two minutes, followed by a temperature ramp of 5 °C/min until 250 °C, which was held for an additional two minutes. Finally, the temperature was increased at a rate of 30 °C per minute until reaching 300 °C, which was maintained for two minutes. Temperatures in the MS transfer line and injector were set at 260 °C and 270 °C, respectively. Helium was used as the carrier gas with a 1 mL/min flow rate. A diluted sample of 1 μL was automatically injected with a solvent delay of 4 minutes by an Autosampler AS1300 in split mode. Electron ionization (EI) mass spectra were obtained at an ionization voltage of 70 eV, covering the m/z 50–650 range. The ion source was heated to a temperature of 200 °C. The identification of GCS components were performed by cross-referencing their mass spectra with those available in the WILEY 09 and NIST 14 databases (https://www.sisweb.com/software/ms/wiley.htm).

2.2. Preparation of AFB1

The *Aspergillus flavus* MD 341 strain was obtained from the Laboratory of Residues of Agricultural Products, Agriculture Pesticides Residues Centre, Dokki, Egypt. The strain was macroscopically and microscopically identified by a mycologist at the center, following the protocols of Pitt et al. (1983) and Varga et al. (2011) after five and seven days of culturing on specific media. AFB1 production by the fungus was achieved by growing it for eight days at 25–27 °C in a sterile synthetic liquid medium containing 20 % sucrose and 2 % yeast extract. The concentration of AFB1 in the medium was determined using high-performance liquid chromatography (HPLC) according to the AOAC (2005) protocol (Fig. S1). AFB1 was the sole component of the medium and was subsequently mixed with the feed to get the desired concentration of AFB1.

2.3. Experimental fish

The feeding experiment was conducted at the Fish Diseases and Management Department, Faculty of Veterinary Medicine, Zagazig University, Egypt. Nile tilapia (*Oreochromis niloticus*) weighing 25.14 ± 0.39 g were obtained from the fish rearing unit at the Faculty of Veterinary Medicine, Zagazig University, Egypt. The fish were acclimatized to the laboratory conditions for two weeks prior to the experiment. They were fed a basal diet and raised in 75 L glass aquariums (30×40×80 cm) filled with dechlorinated water and provided with continuous aeration from an air stone connected to a central air compressor. The fish were subjected to a controlled photoperiod of 12 hours light and 12 hours dark using fluorescent ceiling lights (06:00–18:00). Each aquarium's water was partially replaced daily with clean, dechlorinated water at 8:00 am after cleaning and eliminating the accumulated excreta. Throughout the experiment, the water quality parameters in all aquariums were maintained at suitable levels: pH of 7.67 ± 0.38, dissolved oxygen of 6.80 ± 0.31 mg/L, temperature of 26.50 ± 0.37 °C, and total ammonia nitrogen of 0.11 ± 0.01 mg/L. These water quality parameters were appropriate for Nile tilapia rearing (Boyd and Tucker, 2012).

2.4. Diet preparation and experimental scheme

In line with the Nutrient Requirements of Fish (National Research Council, 2011), the experimental diets were formulated to meet the

nutritional needs of the fish, as shown in Table 1. The chemical composition of each diet was done following the AOAC (2005) protocols. The crude protein content was determined by the macro-Kjeldahl method. Crude lipids were determined using petroleum ether 60–80, total ash was quantified using a muffle furnace, and crude fiber was determined after the acid and alkaline digestions. The fish were fed a daily amount of feed equivalent to 3 % of their body weight, divided into three feedings at 9:00, 12:00, and 16:00 h, to prevent overfeeding and ensure proper digestion and protein utilization (Riche et al., 2004). Each group consisted of three replicates of fifteen fish, totaling two hundred and seventy fish split into six groups. The 1st group was fed a basal diet without any supplementation. The 2nd and 3rd groups were fed diets containing 1 % or 2 % GCS, respectively. The selection of these GCS levels was based on a previous study by Gharieb (2017), which demonstrated the beneficial effects of including 1 % GCS in the diet of *O. niloticus*. To mitigate the adverse effects of AFB1 contamination-induced stress, we incorporated GCS at concentrations of 1 % and 2 %. The 4th group was fed an AFB1-contaminated diet at 2 mg/kg. The AFB1 concentration was chosen based on the findings of Deng et al. (2010) and Ayyat et al. (2018), which showed that feeding diets containing AFB1 at a dose of 2 mg/kg decreased growth performance indicators, feed efficiency, and overall health and increased the amount of AFB1 residue in the blood of juvenile tilapia. To prepare the AFB1 diet, 2 mL of warm chloroform was used to dissolve AFB1. The mixture was then thoroughly diluted with 50 mL of fish oil until completely dissolved. Next, a 60 °C water bath was established within a fume hood. The mixture of fish oil and AFB1 solution was introduced into the water bath, facilitating the complete evaporation of chloroform. After collecting the toxic fish oil, it was added to fresh fish oil per the intended concentration gradient. The feed ingredients were carefully mixed with fresh and toxic fish oils during diet preparation. The 5th and 6th groups were fed a GCS and AFB1-containing diet at the previously mentioned levels. We thoroughly mixed each diet's components and then added 100 mL of water/kg diet. The final paste for each diet was made using a standard kitchen blender. The diets were then pelletized using a laboratory pellet machine and dried for 24 hours before being stored at 4 °C in plastic bags until use. The feeding trial lasted for 60 days.

Table 1
Ingredients and chemical analysis of the experimental diets (g kg^{-1} diet).

	Experimental diets					
	Control	GC1 %	GC2 %	AFB1	AFB1+ GCS1 %	AFB1+ GCS2 %
Ingredients						
Fish meal	110	110	110	110	110	110
Corn flour	330	330	330	330	330	330
Soybean meal 44 %	290	290	290	290	290	290
Corn gluten meal 60 %	120	120	120	120	120	120
Wheat bran	80	70	60	79.998	69.998	59.998
Soybean oil	20	20	20	20	20	20
Fish oil	20	20	20	20	20	20
Garden cress seeds (GCS)	0	10	20	0	10	20
Aflatoxin B1	0	0	0	0.002	0.002	0.002
Vitamin premix[a]	15	15	15	15	15	15
Mineral premix[b]	15	15	15	15	15	15
Total	1000	1000	1000	1000	1000	1000
Chemical analysis						
Crude protein (N ×6.25)	305.7	306.9	308.1	305.8	306.7	308.5
Crude lipids	74.9	77.5	80.2	75.0	77.5	80.4
Crude fiber	52.7	52.6	52.5	52.4	52.1	52.9
Ash	53.2	53.6	54.0	53.6	53.0	54.5
Nitrogen free extract[c]	513.5	509.4	505.2	513.2	510.7	503.7
Gross energy (MJ/kg)[d]	19.02	19.08	19.14	19.02	19.10	19.14

[a] Vitamin premix (per kg of premix): vitamin A, 8,000,000 IU; vitamin E, 7000 mg; vitamin D_3, 2,000,000 IU; vitamin K_3,1500 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20,000 mg; pantothenic acid,7000 mg; vitamin B_1, 700 mg; vitamin B_2, 3500 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 7 mg.

[b] Mineral premix (per kg of premix): zinc sulfate, 4.0 g; iron sulfate, 20 g; manganese sulfate, 5.3 g; copper sulfate, 2.7 g; calcium iodine, 0.34 g; sodium selenite, 70 mg; cobalt sulfate, 70 mg, and $CaHPO_4 \cdot 2\ H_2O$ up to 1 kg.

[c] Calculated by difference (1000 – protein+ lipids + ash + crude fiber).

[d] Gross energy (GE) was calculated as 23.64, 39.54, and 17.20 kJ/g for protein, lipid, and NFE, respectively (NRC, 1993).

2.5. *Growth performance and mortalities*

The mortality rate was determined by dividing the number of dead fish by the total initial number of fish. Initial body weight (IBW) was calculated at the feeding trial's beginning and end as final body weight (FBW). The weight gain (WG), daily weight gain (DWG), condition factor (K), specific growth rate (SGR) and were estimated following formulas:

WG = FBW (g) - IBW (g)

DWG = Weight gain/time intervals (days)

SGR = 100 × ((Ln FBW (g) - Ln IBW (g))/time intervals (days).

K-factor = (W×100)/L3, where W and L refer to the fish's body weight and length, respectively.

2.6. *Collection of samples*

After the 60-day feeding trial, two sets of blood samples were taken from the caudal veins of six fish/replicates sedated with a solution of clove solution (200 mg/L). The first set of samples was collected into EDTA tubes for blood cell count, while the second set was collected in anticoagulant-free tubes, allowed to clot overnight at 4 °C, and then centrifuged at 3500 g for 15 minutes to obtain serum. The serum was used to assess protein electrophoretic pattern, immunological function, liver and kidney functions, and oxidants/antioxidants status. Following blood sampling, the fish were dissected, and the kidney, liver, small intestine, and spleen were collected and kept in 10 % neutral buffered formalin for histopathological assessments. Muscle samples were also collected from each group to analyze AFB1 residues.

2.7. *Hematological indices evaluation*

Complete blood counts were performed at the Animal Health Research Institute, Ministry of Agriculture, Zagazig, using an automatic cell counter (XE-2100 Sysmex, Kobe, Japan) in accordance with the method of Fazio et al. (2012) to determine total and differential leukocyte counts, erythrocytes count (RBCs), packed cell volume (PCV), mean corpuscular volume (MCV), hemoglobin level (Hb), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH).

2.8. *Biochemical analysis of the serum*

2.8.1. *Liver and kidney function parameters*

Serum samples were analyzed for aspartate aminotransferase (Ast), alkaline phosphatase (Alp), and alanine aminotransferase (Alt) activities using Spinreact kits (Esteve De Bas, Girona, Spain) in accordance with the protocols of Burtis and Ashwood (1994), Murray (1984), and Wenger et al. (1984), respectively. The bilirubin serum level was determined in line with Walters and Gerarde (1970). Urea and creatinine were measured using Spinreact kits (Esteve De Bas, Girona, Spain) following the procedures outlined by Kaplan (1984) and Fossati et al. (1983), respectively. Electrophoresis was performed to analyze total serum proteins, globulins, and albumin, following the protocol by

Badawi (1971).

2.8.2. The non-specific immune analyses

Lysozyme activity was determined using the turbidimetric technique (Ellis, 1990) with a *Micrococcus lysodeikticus* suspension (Sigma-Aldrich, USA). A Gram-positive bacterium sensitive to lysozyme lysis was used to perform the test (*M. lysodeikticus*). Complement 3 and nitric oxide (NO) concentrations were measured following the manufacturer's instructions using ELISA kits (MyBioSource, San Diego, USA).

2.8.3. Evaluation of enzymatic antioxidants and lipid peroxidation marker

Serum activity of catalase (Cat), glutathione peroxidase (Gpx), superoxide dismutase (Sod), and malondialdehyde (MDA) concentration were estimated by MyBioSource fish ELISA kits (San Diego, USA). The specific catalog numbers for the kits were MBS779072, MBS038818, MBS705700, and MBS1601664, respectively. The manufacturer's instructions were followed for all measurements.

2.8.4. Stress status assays

Cortisol serum levels were determined by a specific ELISA kit (Catalog No. OKEH02541, Aviva Systems Biology Co., USA), according to the manufacturer's instructions. Inter- and intra-assays for cortisol measurement were 4.0 % and 6.1 %, respectively. Glucose levels were measured using a calorimetric kit obtained from Diamond Diagnostic Co., Egypt, following the manufacturer's guidelines.

2.9. Determination of AFB1 residues

Muscle samples from three fish in each experimental group were homogenized in 30 mL of a water/methanol solution (20:80, v/v). After 10-minute centrifugation at 1500 ×g, 15 mL of the supernatant was mixed 1:1 with an 80:20 v/v water/methanol solution. Each sample was vortexed after adding an internal standard of AFB1 labeled with an isotope at a concentration of 5 ng/mL. After filtering the samples through a 0.2-μm Whatman filter, the quantities of AFB1 were measured using HPLC. At a flow rate of 1 mL/min, the mobile phase (methanol 45 %) was injected into the HPLC system. The column temperature was set to 40 °C, and the analyses were carried out with a fluorescence detector. The AFB1 reference material was bought from Sigma-Aldrich in St. Louis, Missouri, USA.

2.10. Histopathological study

After 60 days, the fish were euthanized by decapitation, and representative tissue samples from the kidneys, livers, spleens, and intestines of fifteen fish per group (five fish per replicate) were collected. The samples were rinsed with distilled water and fixed in 10 % phosphate-buffered formalin solution for three days. Subsequently, they were washed again with distilled water, dehydrated using a series of graded ethanol (70–100 %), cleared with xylene, embedded in paraffin wax, sectioned at 5-μm slices, and stained with hematoxylin and eosin, as described by Suvarna et al. (2018). They were then microscopically inspected. Following the technique proposed by Bernet et al. (1999), the recorded histopathological changes in all experimental groups were quantitatively evaluated. The kidney, liver, spleen, and intestinal indices were computed. Seventy-five images per group (five arbitrarily chosen non-overlapping microscopic fields per organ per fish, using a 10 objective) were analyzed. Liver, spleen, kidney, and intestinal parameters were determined after examining imaging data Higher indices indicate poorer histopathological condition of the organs. Histopathological alterations in the kidney, liver, spleen, and intestine were categorized based on the nature of the alteration into five reaction patterns (each pattern includes a group of histologically associated alterations): inflammatory, circulatory, progressive, regressive, and neoplastic. Kidney and liver histological indices were calculated using the formula: Organ index (I_{org}) = $\Sigma_{rp} \Sigma_{alt} (a_{org\ rp\ alt} \times w_{org\ rp\ alt})$.

Where rp= reaction pattern, alt= histopathological change, a= score value (represents the severity of the alteration and can range from 0 (no alteration) to 6 (extensive lesion), and w= importance factor [indicates the changing severity and can range between three (great importance) and one (least importance)]. Based on this method, the overall index for each fish was equal to the sum of the indices for its liver, kidneys, spleen, and intestine, consistent with the following formula: Total index (Tot-I) = $\Sigma_{rp} \Sigma_{org} \Sigma_{alt} (w_{org\ rp\ alt} \times a_{org\ rp\ alt})$. Additionally, a multiparametric morphological analysis of the intestinal villus height, villus width (villus apical width + villus basal width)/2, and villus surface area villus height × villus width) were calculated in ten villi/fish consistent with Sun et al. (2005). All dimensions were performed by the AmScope ToupView V3.7.13522 software, AmScope, USA.

2.11. Challenge test

A culture of *Aeromonas hydrophila* was obtained from moribund fish in the Fish Diseases and Management Department. Fish organs (liver, spleen, and kidney) were sampled under strict hygienic environments. The inoculums were spread out onto tryptic soya agar plates (TSA; Difco, Detroit, MI), where they were incubated for 24 hours at 37 °C. Then, a single colony was picked from each plate to be sub-cultured in a new dish of Rimler-Shotts agar medium (RS; Difco, Detroit, MI). The isolates were categorized by the routine study of the morphological character, biochemical reactions, an API 20NE test kit (bioMérieux, Marcy l'Etoile, France), and the VITEK® C15 automated system for identification of the bacteria (BioMerieux Inc., France) following manufacturer's directions. After 60 days, 0.2 mL of pathogenic *A. hydrophila* (24 h old culture, 1×10^8 CFU) was intraperitoneally injected into 15 fish from each group. The selected dose corresponded to median lethal dose (LD_{50}) determined by Ashour et al. (2020) for the same speciesThe LD_{50} caused 50 % fish mortality was 2.8×10^8 CFU/mL. The bacterial challenge test was utilized a sub-lethal dose. After injection, the challenged fish were observed daily for two weeks to record any abnormal behavior, clinical signs, and mortalities. The average mortality in all experimental groups was computed to assess the relative percent survival (RPS) (Amend, 1981). *A. hydrophila* was re-isolated and verified from clinically sick and freshly dead fish. *A. hydrophila* was re-isolated and confirmed from clinically sick and freshly dead fish using biochemical characterization, Gram staining, and colonial features. The biochemical characteristics of the bacterial isolates were checked using the VITEK® compact (BioMérieux).

2.12. Statistical analysis

The data were presented as means ± standard error of the mean. Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, USA) and a Two-way ANOVA. Differences between means were determined using Tukey's Multiple Range Test at a significance level of 0.05. The Shapiro-Wilk W test and Levene's test were used to assess the normal distribution and homogeneity of the data, respectively. The parameters that did not meet the assumptions of two-way ANOVA underwent a log transformation, but untransformed data are presented. If the transformations proved ineffective, the nonparametric Kruskal-Wallis H test was used instead.

3. Results

3.1. GC-MS profile of GCS

Table 2 and Fig. S2 displayed the GCS components, their retention times, and the relative percentage of the total peak area resulting from the GC-MS investigation. A total of 16 signals have been identified. The major components of the GCS were benzene, (isothiocyanatomethyl)- (33.93 %), 3′,5′-dimethoxyacetophenone (20.92 %), 2-phenylacetonitrile (13.50 %), cholestan-3-ol, 2-methylene-,(3á,5à)- (7.12 %), ç-

Table 2
Retention time (RT) and peak area (%) of the different compounds found in garden cress (*Lepidium sativum*) seeds analyzed by GC-MS.

Peak number	Compound	RT (min)	Peak area %	Molecular formula	Molecular weight
1	Benzene, (isothiocyanatomethyl)-	13.64	33.93	C_8H_7NS	149
2	3',5′-Dimethoxyacetophenone	18.43	20.92	$C_{10}H_{12}O_3$	180
3	2-Phenylacetonitrile	8.11	13.50	C_8H_7N	117
4	Cholestan-3-ol, 2-methylene-,(3á,5à)-	30.36	7.12	$C_{28}H_{48}O$	400
5	ç-Tocopherol	42.94	5.66	$C_{28}H_{48}O_2$	416
6	á-Sitosterol	36.14	4.19	$C_{29}H_{50}O$	414
7	9-Octadecenoic Acid (*Z*)-, 2-Hydroxy-1-(Hydroxymethyl) Ethyl Ester	39.21	2.80	$C_{21}H_{40}O_4$	356
8	Sinapic acid methyl ester	29.24	2.60	$C_{12}H_{14}O_5$	238
9	Campesterol	45.23	2.60	$C_{28}H_{48}O$	400
10	Pentadecanoic acid	27.06	1.38	$C_{15}H_{30}O_2$	242
11	Benzeneacetonitrile, 3-chloro-	10.16	1.08	C_8H_6ClN	151
12	Ethyl iso-allocholate	43.69	0.98	$C_{26}H_{44}O_5$	436
13	9-Octadecenoic acid, Methyl ester	29.49	0.96	$C_{19}H_{36}O_2$	296
14	2,2,3,3,4,4 Hexadeutero octadecanal	35.56	0.94	$C_{18}H_{30}D_6O$	274
15	Benzenemethanol, à-[1-(Methylamino)Ethyl]-, [S-(R*,R*)]-	16.35	0.77	$C_{10}H_{15}N$	149
16	1-Heptatriacotanol	39.82	0.58	$C_{37}H_{76}O$	536

tocopherol (5.66 %), á-Sitosterol (4.19 %), 9-Octadecenoic Acid (*Z*)-, 2-hydroxy-1-(Hydroxymethyl) ethyl ester (2.80 %), Sinapic acid methyl ester (2.60 %), and campesterol (2.60 %).

3.2. Changes in fish growth and survival

The impact of adding GCS and/or AFB1 to the diet of *O. niloticus* on the fish growth performance for 60 days is presented in Table 3. Throughout the trial period (0−60 days), AFB1 contamination significantly ($p < 0.001$) reduced various growth parameters, including BWG, FBW, WG %, SGR, DWG, and K-factor. Additionally, fish survival (p=0.002) was significantly reduced in the AFB1-contaminated groups (AFB0) compared to non-contaminated fish groups. However, the addition of 1 % GCS and 2 % GCS resulted in a significant ($p < 0.001$) enhancement in fish survival BWG, FBW, WG %, SGR, DWG, and K-factor. The highest growth rate was observed when 2 % GCS was added to the fish diet. The interaction between GCS supplementation and AFB1 contamination significantly influenced the growth and survival of *O. niloticus*.

3.3. Effects on hematological indices

AFB1 contamination significantly ($p < 0.001$) reduced RBCs, PCV %, Hb, MCHC, WBCs, heterophils, lymphocytes, monocytes, and eosinophil levels, and increased MCV compared to the non-contaminated groups (Table 4). Conversely, GCS supplementation significantly ($p < 0.001$) increased Hb, RBCs, PCV %, MCHC, WBCs, heterophils, lymphocytes, and eosinophil levels, while decreasing MCV compared to the non-supplemented diet. However, GCS supplementation did not significantly affect the monocyte count. The interaction between GCS supplementation and AFB1 contamination significantly influenced all measured hematological parameters except eosinophil count (Table 4).

3.4. Effects on liver function indices, protein profile, and renal damage products

The changes in serum enzymes, bilirubin, kidney damage products, and protein profile in fish-fed AFB1 and/or GCS-containing diets for 60 days are presented in Table 5. AFB1 contamination significantly ($p < 0.001$) increased the levels of serum enzymes (Alp, Alt, and Ast), bilirubin, creatinine, and urea. In contrast, the inclusion of GCS in the diet

Table 3
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on growth performance and survival of *Oreochromis niloticus* fed aflatoxin B1 (AFB1) containing diet exposed for 60 days.

Parameter	AFB level	GCS supplementation level			Two-way ANOVA (P-value)		
		GCS0	GCS1	GCS2	AFB1	GCS	Interaction
Initial body weight, g	AFB0	25.30 ± 0.29	25.23 ± 0.29	25.13 ± 0.19	0.439	0.802	0.975
	AFB2	25.17 ± 0.23	25.00 ± 0.32	25.00 ± 0.17			
Final body weight, g	AFB0	53.47^b ± 0.49	57.83^a ± 0.66	60.33^a ± 1.11	<0.001	<0.001	0.027
	AFB2	40.57*^{c} ± 0.83	46.47*^{b} ± 0.32	52.20*^{a} ± 0.93			
Final length, cm	AFB0	15.27 ± 0.18	15.50 ± 0.10	15.50 ± 0.06	0.219	0.297	0.297
	AFB2	15.30 ± 0.12	15.17 ± 0.12	15.43 ± 0.09			
Weight gain, g	AFB0	28.17^b ± 0.20	32.60^a ± 0.38	35.20^a ± 0.93	<0.001	<0.001	0.004
	AFB2	15.40*^{c} ± 0.60	21.47*^{b} ± 0.07	27.20*^{a} ± 0.76			
Weight gain %	AFB0	111.0^c ± 0.58	128.7^b ± 0.33	139.7^a ± 2.60	<0.001	<0.001	0.001
	AFB2	61.10*^{c} ± 1.85	85.83*^{b} ± 1.18	108.3*^{a} ± 2.33			
Daily weight gain, g	AFB0	0.47^b ± 0.003	0.54^a ± 0.01	0.59^a ± 0.02	<0.001	<0.001	0.005
	AFB2	0.26*^{c} ± 0.01	0.36*^{b} ± 0.004	0.45*^{a} ± 0.01			
Specific growth rate, %	AFB0	0.54^c ± 0.002	0.60^b ± 0.003	0.63^a ± 0.01	<0.001	<0.001	<0.001
	AFB2	0.34*^{c} ± 0.01	0.45*^{b} ± 0.003	0.53*^{a} ± 0.01			
Condition factor	AFB0	1.50 ± 0.04	1.55 ± 0.02	1.62 ± 0.01	<0.001	<0.001	0.002
	AFB2	1.13*^{c} ± 0.01	1.33*^{b} ± 0.03	1.41*^{a} ± 0.01			
Survival %	AFB0	96.67 ± 3.33	100.00 ± 0.00	100.00 ± 0.00	0.002	<0.001	0.002
	AFB2	70.00*^{b} ± 5.77	96.67^a ± 3.33	100.00^a ± 0.00			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels (p < 0.05; n = 3 replicates, 15 fish/replicate). Values are represented as the mean ±SEM. Survival data was arcsine transformed before ANOVA analysis to ensure normality and homogeneity of variance. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg−1 diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

Table 4
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on hematological indices of *Oreochromis niloticus* fed aflatoxin B1 (AFB1) containing diet exposed for 60 days.

Parameter	AFB1 level	GCS supplementation level			Two-way ANOVA (P-value)		
		GCS0	GCS1	GCS2	AFB1	GCS	Interaction
RBCs ($\times10^6$/μL)	AFB0	$2.67^{b} \pm 0.07$	$2.83^{ab} \pm 0.05$	$2.99^{a} \pm 0.06$	<0.001	<0.001	<0.001
	AFB2	$1.19^{*c} \pm 0.01$	$2.02^{*b} \pm 0.04$	$2.68^{*a} \pm 0.06$			
Hb (g/dL)	AFB0	$6.99^{c} \pm 0.09$	$7.41^{b} \pm 0.04$	$7.95^{a} \pm 0.12$	<0.001	<0.001	<0.001
	AFB2	$3.65^{*c} \pm 0.03$	$5.06^{*b} \pm 0.06$	$6.91^{*a} \pm 0.08$			
PCV (%)	AFB0	$24.27^{b} \pm 0.43$	$25.47^{b} \pm 0.15$	$27.20^{a} \pm 0.38$	<0.001	<0.001	<0.001
	AFB2	$13.12^{*c} \pm 0.04$	$17.72^{b} \pm 0.19$	$23.82^{a} \pm 0.27$			
MCV (fl)	AFB0	90.91 ± 3.23	90.15 ± 1.56	91.17 ± 2.69	0.01	<0.001	<0.001
	AFB2	$110.55^{*a} \pm 0.73$	$87.89^{b} \pm 1.09$	$88.79^{b} \pm 0.85$			
MCH (pg/cell)	AFB0	26.18 ± 0.74	26.23 ± 0.45	26.66 ± 0.77	0.06	<0.001	<0.001
	AFB2	$30.75^{*a} \pm 0.05$	$25.12^{b} \pm 0.30$	$25.76^{b} \pm 0.23$			
MCHC (%)	AFB0	28.81 ± 0.20	29.09 ± 0.01	29.22 ± 0.04	<0.001	<0.001	0.019
	AFB2	$27.84^{*b} \pm 0.18$	$28.58^{a} \pm 0.02$	$29.00^{a} \pm 0.02$			
WBCs ($\times10^3$/μL)	AFB0	4.58 ± 0.15	4.81 ± 0.06	4.92 ± 0.03	<0.001	<0.001	<0.001
	AFB2	$2.15^{*c} \pm 0.03$	$3.65^{*b} \pm 0.12$	$4.23^{*a} \pm 0.07$			
L ($\times10^3$/μL)	AFB0	$2.22^{b} \pm 0.07$	$2.36^{ab} \pm 0.08$	$2.62^{a} \pm 0.04$	<0.001	<0.001	0.01
	AFB2	$1.21^{*b} \pm 0.07$	$1.75^{*a} \pm 0.06$	$2.03^{*a} \pm 0.05$			
H ($\times10^3$/μL)	AFB0	1.22 ± 0.07	1.36 ± 0.03	1.37 ± 0.05	<0.001	<0.001	0.003
	AFB2	$0.56^{*b} \pm 0.04$	$0.97^{*a} \pm 0.01$	$1.07^{*a} \pm 0.03$			
E ($\times10^3$/μL)	AFB0	0.35 ± 0.05	0.39 ± 0.05	0.42 ± 0.04	<0.001	0.013	0.194
	AFB2	$0.15^{*b} \pm 0.04$	$0.31^{a} \pm 0.02$	$0.36^{a} \pm 0.04$			
M ($\times10^3$/μL)	AFB0	0.79 ± 0.08	0.71 ± 0.12	0.51 ± 0.04	0.03	0.076	<0.001
	AFB2	$0.23^{*b} \pm 0.04$	$0.62^{a} \pm 0.03$	$0.78^{a} \pm 0.04$			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels ($p < 0.05$; n = 3 replicates, 5 fish/replicate). Values are represented as the mean ±SEM. RBCs: red blood cells; Hb: hemoglobin; PCV: packed cell volume; MCV: Mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBCs: white blood cells; L: Lymphocytes; H: Heterophils; E: Eosinophils; M: Monocytes. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg−1 diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

Table 5
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on liver and kidney function indicators, total protein profile, immunological function, and stress indicators of *Oreochromis niloticus* fed aflatoxin B1 (AFB1) containing diet exposed for 60 days.

Parameter	AFB1 level	GCS supplementation level			Two-way ANOVA (P-value)		
		GCS0	GCS1	GCS2	AFB1	GCS	Interaction
Alt (U/L)	AFB0	13.67 ± 0.55	12.53 ± 0.32	12.70 ± 0.32	<0.001	<0.001	<0.001
	AFB2	$75.27^{*a} \pm 2.95$	$32.17^{*b} \pm 1.30$	$14.37^{c} \pm 0.69$			
Ast (U/L)	AFB0	26.80 ± 0.32	26.17 ± 0.33	26.13 ± 0.35	<0.001	<0.001	<0.001
	AFB2	$62.97^{*a} \pm 1.60$	$37.50^{*b} \pm 1.44$	$27.77^{c} \pm 0.41$			
Alp (U/L)	AFB0	38.20 ± 0.40	37.37 ± 0.27	37.63 ± 0.27	<0.001	<0.001	<0.001
	AFB2	$50.27^{*a} \pm 0.67$	$41.23^{*b} \pm 0.76$	$38.80^{c} \pm 0.15$			
Bilirubin (mg/dL)	AFB0	0.29 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	<0.001	<0.001	<0.001
	AFB2	$1.00^{*a} \pm 0.01$	$0.48^{*b} \pm 0.01$	$0.31^{*c} \pm 0.01$			
Urea (mg/dL)	AFB0	2.83 ± 0.01	2.77 ± 0.01	2.76 ± 0.01	<0.001	<0.001	<0.001
	AFB2	$9.90^{*a} \pm 0.38$	$4.10^{*b} \pm 0.21$	$2.90^{c} \pm 0.02$			
Creatinine (mg/dL)	AFB0	0.50 ± 0.01	0.47 ± 0.01	0.46 ± 0.00	<0.001	<0.001	<0.001
	AFB2	$0.97^{*a} \pm 0.01$	$0.63^{*b} \pm 0.01$	$0.52^{*c} \pm 0.01$			
Total protein (g/dL)	AFB0	5.83 ± 0.12	5.97 ± 0.09	6.20 ± 0.17	<0.001	<0.001	<0.001
	AFB2	$2.63^{*c} \pm 0.15$	$4.17^{*b} \pm 0.20$	$5.40^{*a} \pm 0.12$			
Albumin (g/dL)	AFB0	2.42 ± 0.04	2.42 ± 0.04	2.58 ± 0.09	<0.001	<0.001	<0.001
	AFB2	$1.23^{*c} \pm 0.09$	$1.63^{*b} \pm 0.12$	$2.22^{a} \pm 0.05$			
Globulin (g/dL)	AFB0	3.41 ± 0.09	3.54 ± 0.05	3.62 ± 0.09	<0.001	<0.001	<0.001
	AFB2	$1.40^{*c} \pm 0.06$	$2.53^{*b} \pm 0.09$	$3.18^{*a} \pm 0.06$			
Lysozyme (μg/mL)	AFB0	$15.63^{c} \pm 0.23$	$17.60^{b} \pm 0.26$	$19.47^{a} \pm 0.32$	<0.001	<0.001	<0.001
	AFB2	$5.87^{*c} \pm 0.20$	$12.70^{*b} \pm 0.25$	$15.33^{*a} \pm 0.26$			
C3 (ug/mL)	AFB0	105.33 ± 1.07	109.10 ± 0.21	111.47 ± 1.07	<0.001	<0.001	<0.001
	AFB2	$55.30^{*c} \pm 2.74$	$90.48^{*b} \pm 2.89$	$103.87^{a} \pm 0.73$			
NO (μmol/L)	AFB0	$49.90^{b} \pm 0.81$	$51.37^{ab} \pm 0.45$	$53.43^{a} \pm 1.09$	<0.001	<0.001	<0.001
	AFB2	$20.63^{*c} \pm 0.78$	$36.60^{*b} \pm 0.59$	$48.27^{*a} \pm 0.46$			
Cortisol (ng/mL)	AFB0	$54.27^{a} \pm 1.11$	$51.27^{ab} \pm 0.71$	$46.27^{b} \pm 1.11$	<0.001	<0.001	<0.001
	AFB2	$87.60^{*a} \pm 1.47$	$58.43^{*b} \pm 1.09$	$56.27^{*b} \pm 1.05$			
Glucose (mg/dL)	AFB0	$76.10^{a} \pm 0.95$	$72.20^{ab} \pm 1.04$	$70.07^{b} \pm 1.43$	<0.001	<0.001	<0.001
	AFB2	$107.30^{*a} \pm 1.14$	$84.03^{*b} \pm 0.90$	$76.73^{*c} \pm 0.85$			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels ($p < 0.05$; n = 3 replicates, 5 fish/replicate). Values are represented as the mean ±SEM. Alt: alanine transaminase; Ast: aspartate transaminase; Alp: alkaline phosphatase; C3: complement 3. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg−1 diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

significantly ($p < 0.001$) lowered the serum levels of Ast, Alp, Alt, bilirubin, urea, and creatinine. However, the total protein, globulin, and albumin levels were higher than fish fed a non-fortified diet. The interaction between GCS supplementation and AFB1 contamination significantly ($p < 0.001$) affected the serum levels of Ast, Alt, urea, creatinine, protein, globulin, and albumin.

3.5. Changes in innate immunity indices

The impact of AFB1 and/or GCS on *O. niloticus* non-specific immunological indicators after 60 days is shown in Table 5. Fish fed AFB1-contaminated diet exhibited a significant ($p < 0.001$) depletion of lysozyme activity, complement 3, and NO contents compared to non-polluted groups. In contrast, fish-fed GCS-fortified diets showed significantly ($p < 0.001$) higher levels of lysozyme, complement 3, and nitric oxide than non-supplemented diets. The interaction between GCS supplementation and AFB1 contamination had a significant effect on all measured innate immune components.

3.6. Effects on stress biomarkers

Cortisol and glucose significantly augmented in fish-fed AFB1-containing diets compared to those fed non-contaminated diets (Table 5). However, GCS supplementation significantly ($p < 0.001$) decreased cortisol and glucose serum levels compared to the non-fortified diets. The interaction between AFB1 contamination and GCS supplementation had a significant ($p < 0.001$) effect on cortisol and glucose blood levels.

3.7. Effects on antioxidant enzymes and lipid peroxidation biomarker

The fish-fed AFB1-contaminated diets displayed a significant ($p < 0.001$) depletion of antioxidant enzymes (Gpx, Sod, and Cat) and increased lipid peroxidation product (MDA) compared to fish-fed non-contaminated diets (Fig. 1). However, the inclusion of GCS in the diet significantly ($p < 0.001$) increased the serum levels of Sod, Cat, and GPx, while reducing MDA content compared to the non-contaminated diets. The interaction between AFB1 contamination and GCS dietary supplementation had a significant impact on lipid peroxidation and oxidative stress parameters in *O. niloticus* (Fig. 1). Notably, despite AFB1 contamination, the highest improvement in Sod and Cat levels was observed when 2 % GCS was added to AFB1-contaminated diets, while there was no significant change in MDA between supplemented groups.

3.8. Histopathological changes

3.8.1. Liver

The livers of non-contaminated groups (AFB0) displayed normal histological structures (Fig. 2A–C). Exposure to AFB1 induced variable degrees of cellular swelling with cytoplasmic vacuolations, hepatocyte hyalinization, spongiosis hepatis, single cell necrosis, coagulative necrotic foci, congestion of the hepatic sinusoids and central veins, minute hemorrhages, mononuclear cell infiltration and/or aggregations and granuloma formations. No cancerous alterations were noticed, but interestingly, hyperplasia involving hepatocytes (Fig. 2D), cholangiocytes (Fig. 2E), and melanomacrophages (Fig. 2F) were evident. The livers of GCS1 % + AFB1-treated fish revealed a substantial decline in the frequency and severity of the AFB1-induced hepatopathic alterations. The most common findings were fatty change associated with nuclear pyknosis, necrotic foci and/or single-cell necrosis (Fig. 2 G and H), perivascular mononuclear cell aggregations (Fig. 2I), vascular congestion and mild hyperplastic proliferation of the hepatocytes, cholangiocytes, and melanomacrophages. The livers of GCS2 % + AFB1-treated fish showed a substantial decline in all the histopathological alterations induced by the AFB1. The basic alterations noticed in this

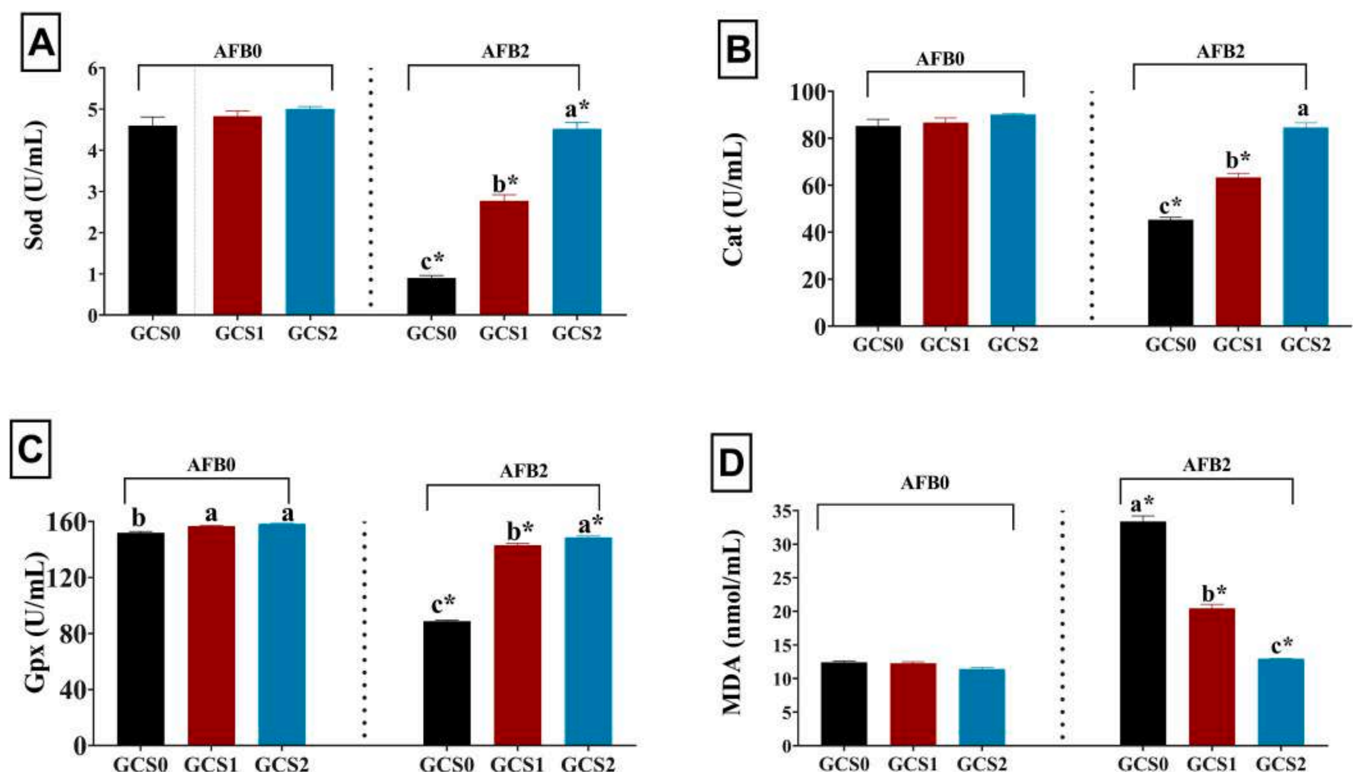

Fig. 1. Effect of garden cress seeds (GCS) supplementation on superoxide dismutase (Sod; **A**), catalase (Cat; **B**), glutathione peroxidase (Gpx; **C**) and malondialdehyde (MDA; **D**) levels in the serum of Nile tilapia fed aflatoxin B1 containing diets for 60 days. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg^{-1} diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS). * indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters indicate a significant difference between GCS levels ($p < 0.05$; $n = 10$ for each group). Data are represented as the mean ± SEM.

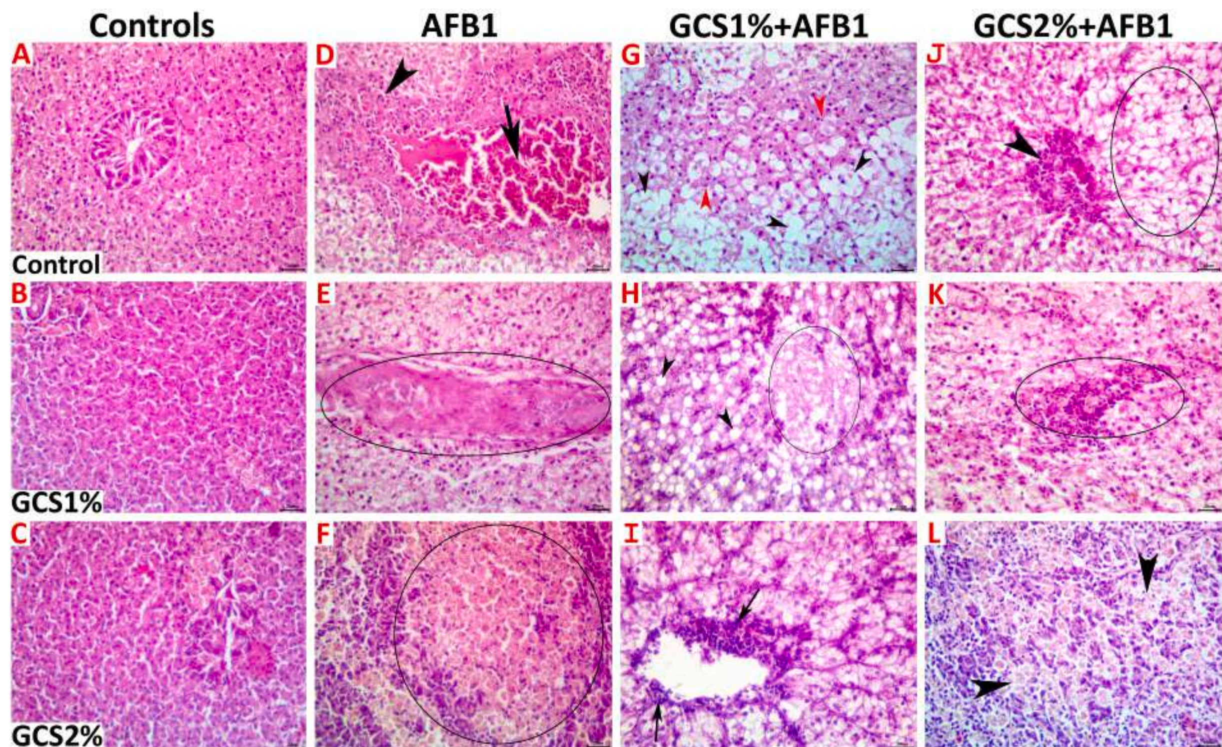

Fig. 2. Representative photomicrograph of H and E-stained hepatic tissue sections showing a normal histological picture in control (A), GCS1 % (B), and GCS2 % (C) treated fish. AFB1-exposed fish showing vascular congestion (red arrow), hyperplasia of hepatocytes (black arrowheads), vascular congestion (black arrow) (D), hyperplasia of cholangiocytes (ellipse) (E), and melanomacrophage aggregates (ellipse) (F). GCS1 % + AFB1-treated fish showing fatty change (black arrowhead), nuclear pyknosis (red arrowheads) (G), necrotic focus (ellipse), fatty change (black arrowheads) (H), and perivascular mononuclear cell aggregation (black arrows) (I). GCS2 % + AFB1-treated fish showing vacuolation focus (black ellipse), mononuclear cell aggregation (black arrowhead) (J), hyperplastic hepatocytes (ellipse) (K), and melanomacrophage aggregates (black arrowheads) (L).

group were vacuolation foci, vascular congestion, and mononuclear cell aggregations (Fig. 2 J). A few specimens showed minute foci of hepatocyte hyperplasia, and one specimen exhibited marked hyperplasia of the melanomacrophage aggregates (Fig. 2 K and L).

3.8.2. Kidneys (posterior kidneys)

The kidneys of non-contaminated groups (AFB0) displayed normal histological structure (Fig. 3A–C). The kidneys of the AFB1 fish showed vascular congestion, tubular vacuolations, epithelial desquamations, cast formations, single-cell necrosis, focal coagulative necrotic foci, and glomerular atrophy with widened Bowman's space, interstitial edema, mononuclear cell infiltrates, and mild fibroblastic proliferation, and hyperplasia of melanomacrophage aggregates (Fig. 3D and E). Few specimens showed glomerular necrosis, tubular cystic dilation, cast formation, and interstitial minute hemorrhages, and the kidneys of two fish showed renal adenomas with no character of malignancy (Fig. 3F). The renal tissues of GCS1 % + AFB1-treated fish exhibited comparable but milder lesions compared to AFB1-exposed fish. The most common lesions were tubular vacuolations, interstitial mononuclear cell infiltrations, single-cell necrosis, and focal necrotic foci (Fig. 3G and H). Renal adenomas were detected in one fish only (Fig. 3I). The renal tissues of GCS1 % + AFB2-treated fish exhibited a significant decline in the frequency and severity of AFB1-induced lesions. The main changes in the earlier group were degenerative and necrotic in the tubular epithelium, sometimes associated with glomerular collapse (Fig. 3H). No neoplastic lesions were detected in this group. The only reported proliferative changes were hyperplasia of the melanomacrophage aggregates and mild hyperplasia of the epithelial lining of a few renal tubules (Fig. 3K and L).

3.8.3. Spleen

The spleens of non-polluted groups (AFB0) revealed normal histology (Fig. 4A and B). Exposure to AFB1 induced lymphoid and erythroid depletion, hyperplasia of the melanomacrophage aggregates vascular, and congestion (Fig. 4C). The spleens of fish exposed to AFB1 and supplemented with either GCS1 % or GCS2 % manifested a sharp reduction in the depletion of the lymphoid and erythroid elements. However, they did not regain their normal histology as some tissue sections in both groups still showed variable grades of vascular congestion and melanomacrophage aggregates hyperplasia (Fig. 4D and E).

3.8.4. Intestine

The intestines of the non-polluted fish groups (AFB0) displayed normal histological pictures (Fig. 4F and G). The intestinal tissue sections of the fish exposed to AFB1 exhibited decreased lymphoid tissue elements and decreased villus width, height, and surface area (Fig. 4H). The intestines of fish exposed to AFB1 and supplemented with either GCS1 % or GCS2 % manifested a slight decrease in the villus height, villus width, and villus surface area compared to the control groups (Fig. 4I and J). Quantitative assessment of the reported lesions in the livers, kidneys, spleens, and intestines of all groups is summarized in Table 6, 7, 8 and 9.

3.9. Effects on residual AFB1 amounts

The results in Table 9 indicate that the presence of AFB1 in the diets led to a tendency to increase (p=0.067) the AFB1 residue in *O. niloticus* compared to non-contaminated diets. However, the addition of GCS to the fish diet significantly (p=0.035) reduced the AFB1 residues compared to the non-fortified diets. The interaction between GCS supplementation and AFB1 contamination significantly (p=0.005) affected

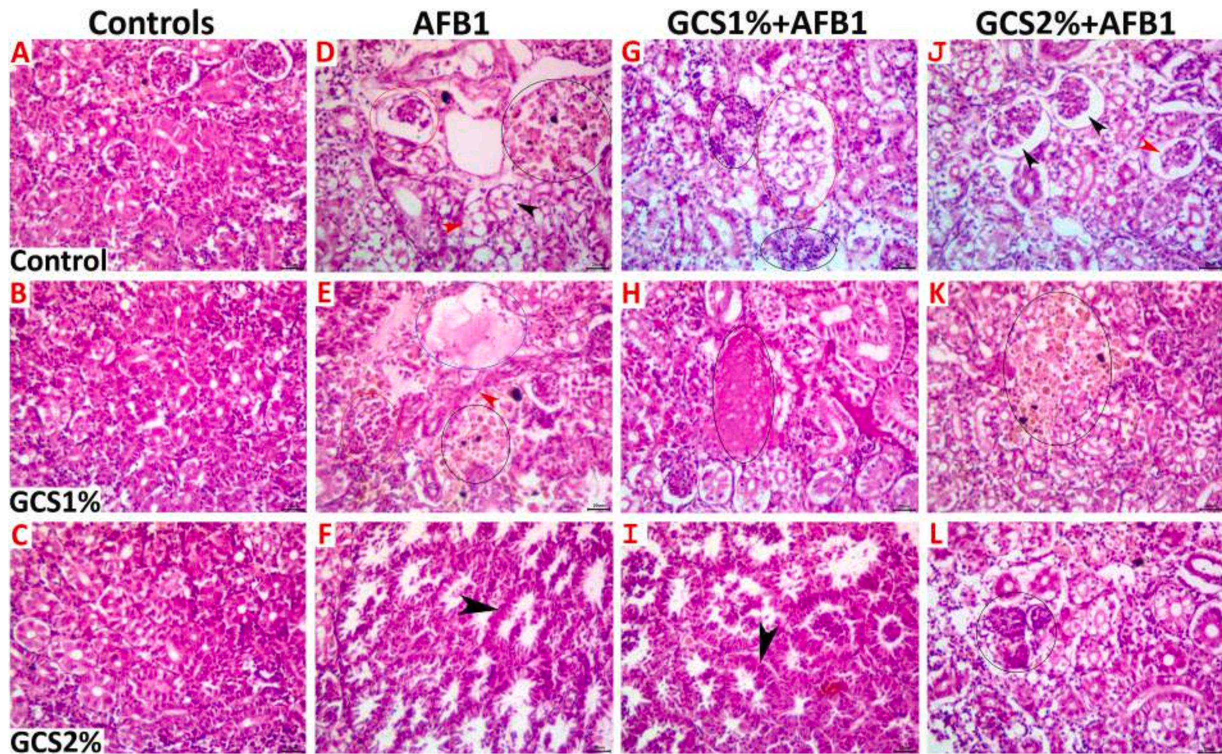

Fig. 3. Representative photomicrograph of H and E-stained renal tissue sections showing a normal histological picture in the normal control (A), GCS1 % (B), and GCS2 % (C) treated fish. AFB1-exposed fish showing glomerular collapse (red ellipse), hyperplastic melanomacrophages (black ellipse), vacuolated (black arrowhead), and necrotic (red arrowhead) tubular epithelium (D), congestion (red ellipse), hyperplastic melanomacrophages (black ellipse), interstitial edema (blue ellipse (E), and tubular adenoma (arrowhead) (F). GCS1 % + AFB1-treated fish showing necrotic renal tubules (red ellipse), interstitial mononuclear cell infiltrations (black ellipses) (G), focal necrotic focus (ellipse) (H), and tubular adenoma (arrowhead) (I). GCS2 % + AFB1-treated fish showing collapsed (blacked arrowheads) and necrotic (red arrowhead) glomeruli (J), hyperplastic melanomacrophages (ellipse) (K), and hyperplastic tubular epithelium (ellipse) (L).

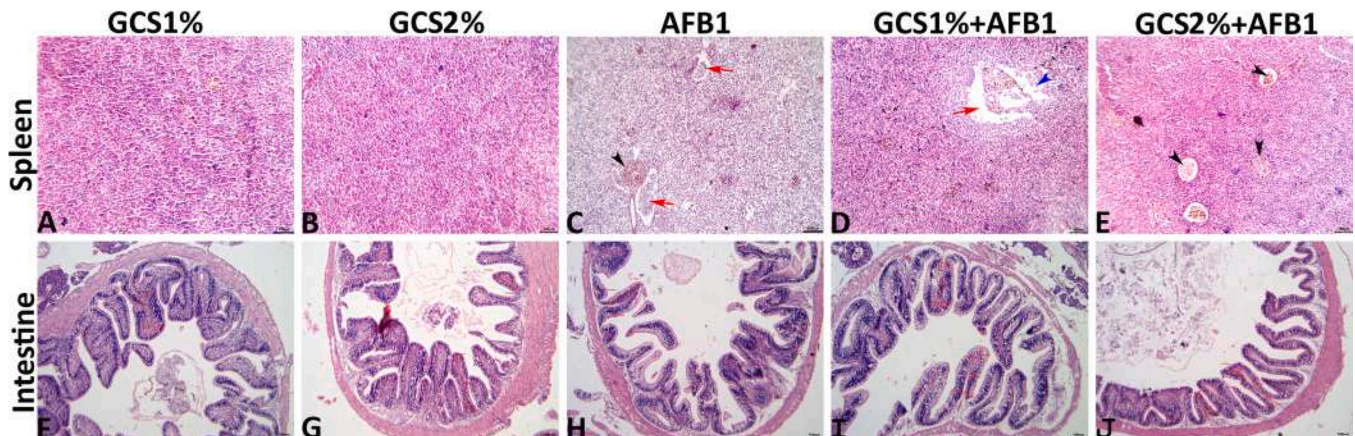

Fig. 4. A–E Representative photomicrograph of H and E-stained splenic tissue sections showing a normal histological picture in the GCS1 % (A) and GCS2 % (B) treated fish, vascular congestion (red arrows), melanomacrophage aggregates hyperplasia (black arrowhead), and notable lymphoid and erythroid depletion in the AFB1-exposed fish (C). The GCS1 % + AFB1-treated fish showing necrotic focus (blue arrowhead) and vascular congestion (red arrow) (D). The GCS2 % + AFB1-treated fish showing hyperplastic melanomacrophage aggregates (black arrowheads) (E). F-J; Representative photomicrograph of H and E-stained intestinal tissue sections showing the villus height and width in the GCS1 % (F), GCS2 % (G), AFB1 (H), GCS1 % + AFB1(I), and GCS2 % + AFB1(J) treated fish.

the AFB1 residues in fish tissues.

3.10. Changes in disease resistance to A. hydrophila

Fish exposed to AFB1 contamination and challenged with *A. hydrophila* exhibited a significantly ($p < 0.001$) higher mortality rate and lower RPS compared to challenged fish and fed the non-contaminated diets (Table 9). However, compared to fish fed non-supplemented GCS diet, supplementation significantly ($p < 0.001$) decreased mortality and enhanced relative percent survival. The interaction between GCS supplementation and AFB1 feed contamination did not significantly affect mortality or relative survival rates.

4. Discussion

In this study, significant growth retardation in was observed in the fish-fed diet containing AFB1 for 60 days. Similar growth-lowering effects of AFB1 contamination have been observed in various fish species,

Table 6
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on lesion scores of hepatic tissues of *Oreochromis niloticus* fed aflatoxin B1 (AFB1) containing diet exposed for 60 days.

Parameter	AFB1 level	GCS supplementation level			Two-way ANOVA (P-value)		
		GCS0	GCS1	GCS2	AFB1	GCS	Interaction
Inflammatory alterations							
Leukocytic infiltration	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.207	<0.001
	AFB2	3.40* ± 0.52	1.80* ± 0.36	1.20* ± 0.33			
Granuloma formation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.005	0.529	0.066
	AFB2	0.60* ± 0.31	0.60* ± 0.31	0.20* ± 0.20			
Circulatory alterations							
Congestion	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.489	<0.001
	AFB2	2.80 * ± 0.42	1.30* ± 0.15	1.20* ± 0.13			
Hemorrhages	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.015	<0.001
	AFB2	1.40*[a] ± 0.22	1.00*[a] ± 0.00	0.20*[b] ± 0.13			
Regressive alterations							
Cellular swelling	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.506	< 0.001
	AFB2	2.60* ± 0.34	1.90*[b] ± 0.23	1.20* ± 0.13			
Vacuolations	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.591	<0.001
	AFB2	3.10* ± 0.57	1.70 * ± 0.33	1.40 * ± 0.22			
Nuclear pyknosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.776	<0.001
	AFB2	1.60* ± 0.16	2.00* ± 0.00	1.60* ± 0.27			
Single cell necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.111	<0.001
	AFB2	3.60* ± 0.40	1.20* ± 0.49	2.70* ± 0.54			
Focal coagulative necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.751	<0.001
	AFB2	4.50 * ± 0.50	3.60* ± 0.40	3.00* ± 0.00			
Progressive alterations							
Fibrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.011	0.026	0.001
	AFB2	0.50*[a] ± 0.17	0.20*[ab] ±0.20	0.00[b] ± 0.00			
Hepatocyte hyperplasia	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.001	0.258	0.004
	AFB2	0.60* ± 0.16	0.40* ± 0.27	0.40* ± 0.27			
Cholangiocyte hyperplasia	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.040	0.768	0.384
	AFB2	0.40* ± 0.27	0.20* ± 0.20	0.20* ± 0.20			
Melanomacrophage hyperplasia	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.630	<0.001
	AFB2	3.80* ± 0.76	2.20* ± 0.76	1.40* ± 0.27			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels using the non-parametric Kruskal-Wallis H test ($p < 0.05$; n = 3 replicates, 5 fish/replicate). Values are represented as the mean ±SEM. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg^{-1} diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

including Nile tilapia (Hussain et al., 2017) and grass carp (Zeng et al., 2019). AFB1 has been shown to bind to naturally occurring gut bacteria that are essential for digestion (Haskard et al., 2001). This binding disrupts the metabolic pathways of gut bacteria, which are crucial for fish health and growth (Talwar et al., 2018). Previous studies have confirmed that AFB1-contaminated diets impair feed conversion ratio in fish (Deng et al., 2010; Sepahdari et al., 2010). AFB1 is also known to impair fish growth by affecting antioxidative and immune responses (Abdel-Daim et al., 2020). In the present study, immunosuppression and oxidative stress were observed in fish AFB1-fed contaminated diet.

Several reports confirmed that the retardation of fish growth due to AFB1 contamination appears to begins in the digestive tract (Barany et al., 2021b, 2021c). Herein, the lowest *villi* height concomitant with the lowest villus surface area recorded in the AFB1-exposed fish may have decreased absorption surface and reduced nutrient utilization, thereby impairing growth. Furthermore, histopathological analysis revealed various abnormalities in the intestinal sections of AFB1-exposed fish, including leukocytic infiltration, hemorrhages, enterocyte necrosis, enterocyte desquamation, and goblet cell hyperplasia. Similar histopathological perturbations have been reported in previous studies investigating the dietary impacts of AFB1 on fish (Andleeb et al., 2015; Hayatullah et al., 2019). Barany et al. (2021c) suggested that AFB1's specific binding to constituents of tight junctions in the gut may be responsible for these gut-architectural impairments. On the other hand, the addition of GCS to the diet of Nile tilapia significantly enhanced growth performance. This growth-promoting activity of GCS may be attributed to its ability to enhance the activity of the digestive enzymes in fish (Bilen et al., 2018). GCS is rich in amino acids, protein, lipids, fiber, moisture, and carbs, which are necessary for fish nutrition (Zia-Ul-Haq et al., 2012). In our experiment, GCS addition to the *O. niloticus* diet significantly maintained the normal intestinal architecture, improved villus height and, enhanced absorptive capacity and nutritional utilization. These factors likely played a role in improving the growth rates of the fish.

Herein, the interaction between GCS dietary supplementation and AFB1 feed contamination had a substantial impact on the survival and growth of *O. niloticus*. Of note, supplementing GCS at a 2 % level significantly enhanced all growth performance indicators and survival in fish-fed diets containing AFB1. In this regard, the GCS's high content of essential fatty acids and essential oils has several benefits for the digestive system, including stimulating the digestive enzymes, restoring microbiota balance, and enhancing nutrient absorption and FCR (El-Saadany et al., 2022). Herein, benzene (isothiocyanatomethyl), a principal constituent of GCS identified through GC-MS analysis, exhibits potent antimicrobial activity against pathogenic gut microbes (Dufour et al., 2013). Additionally, GCS has been reported to efficiently reverse the AFB1-induced oxidative stress and depletion of immune cells (Sanad et al., 2018). Thus, GCS mitigates AFB1-induced growth retardation by counteracting its adverse effects on gut bacterial flora and immune response, and oxidative status.

In the current study, the fish-fed AFB1-contaminated diet exhibited a typical macrocytic normochromic anemia characterized by reduced RBCs, Hb, PCV, and MCHC but increased MCV. In this regard, Verma (2004) demonstrated that aflatoxins including AFB1, might interact with DNA and RNA, inhibiting RBC production. A significant leukopenia was also observed in the AFB1-exposed fish group. In contrast, GCS dietary supplementation effectively counteracted the AFB1-induced anemic and leukopenic conditions. GCS may reverse the AFB1-induced anemia and leukopenia by protecting mitochondrial membrane proteins and phospholipids in RBCs and WBCs from peroxidation and

Table 7
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on lesion scores of renal tissues of *Oreochromis niloticus* fed aflatoxin B1 (AFB1) containing diet exposed for 60 days.

Parameter	AFB1 level	GCS supplementation level			Two-way ANOVA (P-value)		
		GCS0	GCS1	GCS2	AFB1	GCS	Interaction
Inflammatory alterations							
Leukocytic infiltration	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.643	<0.001
	AFB2	3.60* ± 0.50	2.40* ± 0.27	2.00* ± 0.00			
Granuloma formation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.040	0.015	0.001
	AFB2	0.40[a]* ± 0.16	0.00[b] ± 0.00	0.00[b] ± 0.00			
Circulatory alterations							
Congestion	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.439	<0.001
	AFB2	2.80* ± 0.42	1.50 * ± 0.22	1.00* ± 0.00			
Hemorrhage	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.008	<0.001
	AFB2	1.70*[a] ± 0.15	0.60*[a] ± 0.16	0.20*[b] ± 0.13			
Interstitial edema	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.057	<0.001
	AFB2	2.60* ± 0.34	1.20* ± 0.33	0.60* ± 0.27			
Regressive alterations							
Glomerular collapse	AFB0	0.00[b] ± 0.00	0.00[b] ± 0.00	2.30[a] ± 0.50	<0.001	0.011	<0.001
	AFB2	2.50*[a] ± 0.31	1.30*[ab] ± 0.26	1.10*[b] ±0.10			
Glomerular necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.106	<0.001
	AFB2	3.60* ± 0.40	2.40* ± 0.40	1.20* ± 0.49			
Tubular vacuolation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.639	<0.001
	AFB2	3.10* ± 0.53	1.80* ± 0.25	1.50* ± 0.17			
Tubular necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.627	<0.001
	AFB2	4.80* ± 0.49	3.30* ± 0.30	3.00* ± 0.00			
Cystic dilatation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.005	0.079	0.003
	AFB2	0.50* ± 0.17	0.10* ± 0.10	0.10* ± 0.10			
Cast formation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.222	<0.001
	AFB2	1.70* ± 0.26	1.40* ± 0.16	0.70* ± 0.21			
Progressive alterations							
Mesangial proliferation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.021	0.005	<0.001
	AFB2	0.50*[a] ± 0.17	0.00[b] ± 0.00	0.00[b] ± 0.00			
Interstitial fibrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.001	<0.001
	AFB2	1.00*[a] ± 0.15	0.40*[b] ± 0.27	0.00[b] ± 0.00			
Neoplastic alterations	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	<0.001	<0.001
	AFB2	2.40*[a] ± 0.22	0.30*[b] ± 0.30	0.00[b] ± 0.00			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels using the non-parametric Kruskal-Wallis H test ($p < 0.05$; n = 3 replicates, 5 fish/replicate). Values are represented as the mean ±SEM. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg^{-1} diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

preventing oxidative damage to DNA (Abdel-Baky, 2019). In this regard, one of the bioactive compounds identified in GCS, á-sitosterol, has been found to maintained erythrocyte membrane fluidity efficiently (Chen et al., 2020). Another GCS constituent, ç-Tocopherol, has been reported to protect the RBC membrane against oxidative damage (Sovira et al., 2020)

The entry of AFB1 into the bloodstream from the gastrointestinal tract has been documented, leading to detrimental effects on the kidney and liver function (Ahmed et al., 2022).). In our study, fish fed an AFB1-containing diet consistently exhibited elevated levels of bilirubin, Ast, Alt, Alp, creatinine, and urea. Furthermore, significant pathological changes were observed in the liver and kidney tissues of fish exposed to AFB1. Similar hepatic and renal damage due to AFB1 exposure has been reported in Nile tilapia (Hassaan et al., 2020). The hydroxyl radical, a highly destructive reactive oxygen species (ROS), induces lipid peroxidation, resulting in the breakdown of unsaturated fatty acids in cell membranes and the generation of MDA (Marin and Taranu, 2012). Consequently, the increased MDA levels in our study likely contributed to the release of membrane-bound protein ALP, transaminases (Alt and Ast), urea, and creatinine into the bloodstream, thus elevating their serum levels. Interestingly, when GCS was added to the AFB1-contaminated diet, these levels were nearly recovered to normal. This suggests that GCS may play a pharmacodynamics role in regulating liver enzymes and renal function due to its high content of essential amino acids, minerals, vitamins, and fatty acids necessary for optimal metabolic pathways in the liver (Abuelgasim et al., 2008). Additionally, GCS may exhibit hepatoprotective effect owing to its antioxidants and anti-inflammatory components, such as benzene and ç-tocopherol (Miyoshi et al., 2004; Nakamura et al., 2004). Moreover, the presence of dietary phytosterols like á-sitosterol and campesterol in GCS, as detected by GC-MS analysis, has been associated with hepatoprotective activity (Chen et al., 2020).

In our study, fish fed an AFB1-containing diet displayed significant hypoproteinmic, hypoalbuminemia, and hypoglobulinemic. The decrease in total protein and albumin may be attributed to suppressed gene transcription and protein translation resulting from AFB1–8,9-epoxide binding to DNA, which generates AFB1-N7-Guanine adducts and protein adducts. Additionally, the immediate detrimental effect of ROS production contributed to this decline (Coppock et al., 2018). In contrast, tilapia fed with GCS exhibited higher levels of total protein, globulin, and albumin, likely due to enhanced immunity. Furthermore, the reported hepatoprotective activity of GCS may have contributed to the restoration of total protein, albumin, and globulin levels (Raish et al., 2016).

Our study confirmed that fish fed an AFB1-containing diet showed immune-toxic injuries and increased stress, as evidenced by a significant decrease in lysozymes, nitric oxide, and C3 levels, along with elevated cortisol and glucose levels. Moreover, notable pathological changes were observed in the splenic tissues of fish exposed to AFB1. Moreover, the AFB1-induced immunosuppression was highly reflected in the reduced fish survival after *A. hydrophila* challenge. These findings align with previous reports of reduced lysozyme levels in *O. niloticus* following AFB1 exposure (Hassaan et al., 2020), as well as decreased C3 concentrations in various fish species, including Turbot (*Scophthalmus maximus*) and grass carp fed AFB1-contaminated diets (*Ctenopharyngodon idella*) (He et al., 2022; Yang et al., 2020). C3 plays a vital role in

Table 8
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on lesion scores of splenic and intestinal tissues and posterior intestine morphometric indices of *Oreochromis niloticus* fed aflatoxin B1 (AFB1) containing diet exposed for 60 days.

Parameter		AFB1 level	GCS supplementation level			Two-way ANOVA (P-value)		
			GCS0	GCS1	GCS2	AFB1	GCS	Interaction
Splenic tissues	***Circulatory alterations***							
	Congestion	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.021	<0.001
		AFB2	3.00^{*a} ± 0.60	2.00^{*ab} ± 1.33	1.00^{*b} ± 1.00			
	Regressive alterations							
	Lymphoid depletion necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.050	<0.001
		AFB2	8.00^{*a} ± 1.33	3.00^{*ab} ± 1.53	2.00^{*b} ± 1.33			
	Erythroid depletion necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.078	0.045	0.008
		AFB2	1.00^{*a} ± 0.54	0.00^{ab} ± 0.00	0.00^{b} ± 0.00			
	Melanomacrophage aggregates necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.005	0.108	0.005
		AFB2	2.00* ± 0.71	1.00* ± 1.00	1.00* ± 1.00			
	Progressive alterations							
	Melanomacrophage aggregates hyperplasia	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.068	<0.001
		AFB2	4.00* ± 0.54	3.00* ± 1.53	2.00* ± 1.33			
Intestinal tissues	***Inflammatory alterations***							
	Leukocytic infiltration	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.004	<0.001
		AFB2	4.60^{a} ± 0.58	3.00^{a} ± 1.33	0.00^{b} ± 0.00			
	Circulatory alterations							
	Congestion	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.295	<0.001
		AFB2	3.20 ± 0.77	5.00 ± 1.67	2.00 ± 1.33			
	Hemorrhages	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.021	0.005	<0.001
		AFB2	1.00^{a} ± 0.33	0.00^{b} ± 0.00	0.00^{b} ± 0.00			
	Regressive alterations							
	Enterocytes necrosis	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.016	<0.001
		AFB2	5.00^{a} ± 0.98	3.00^{ab} ± 1.53	1.00^{b} ± 1.00			
	Enterocytes desquamation	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.164	0.001
		AFB2	6.00 ± 1.43	3.00 ± 1.53	2.00 ± 1.33			
	Progressive alterations							
	Goblet cell hyperplasia	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.011	0.241	0.032
		AFB2	2.00 ± 0.83	1.00 ± 1.00	1.00 ± 1.00			
	Posterior intestine morphometric indices (μm)							
	Villus height	AFB0	351.90^{a} ± 17.38	359.70^{a} ± 16.99	355.40^{a} ± 17.59	0.002	0.049	0.09
		AFB2	275.80^{b} ± 8.07	320.60^{a} ± 17.19	349.10^{a} ± 13.74			
	Villus width	AFB0	120.70 ± 8.23	121.80 ± 6.86	123.10 ± 8.36	0.345	0.891	0.968
		AFB2	113.90 ± 6.74	118.40 ± 4.78	117.30 ± 5.38			
	Villus surface area	AFB0	43,121 ± 4830	43,937 ± 3419	44,663 ± 4908	0.018	0.271	0.481
		AFB2	31,077 ± 1518	38,461 ± 3255	40,852 ± 2172			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels ($p < 0.05$; n = 3 replicates, 5 fish/replicate). Values are represented as the mean ±SEM. The lesion scores of splenic and intestinal tissues were subjected to statistical analysis using the non-parametric Kruskal-Wallis H test. The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg−1 diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

Table 9
Effect of garden cress (*Lepidium sativum*) seeds (GCS) supplementation on organ indexes and aflatoxin B1 (AFB1) residues of *Oreochromis niloticus* fed AFB1-containing diet exposed for 60 days as well as their mortality rate and relative percent survival after *Aeromonas hydrophila* challenge for 14 days.

Parameter	AFB1 level	GCS supplementation level			Two-way ANOVA (P-value)		
		GCS0	GCS1	GCS2	AFB1	GCS	Interaction
Liver index	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.011	0.241	0.032
	AFB2	28.90* ± 1.32	18.10* ± 1.58	14.70* ± 1.27			
Kidney index	AFB0	0.00 ± 0.00	0.00 ± 0.00	2.30 ± 0.50	<0.001	0.562	<0.001
	AFB2	31.20* ± 1.69	16.70* ± 1.29	11.40* ± 0.78			
Spleen index	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.122	<0.001
	AFB2	18.00* ± 2.66	9.00* ± 3.14	6.00* ± 2.67			
Small intestine index	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	0.164	<0.001
	AFB2	21.80 * ± 3.88	15.00* ± 5.37	6.00* ± 2.67			
Total organ index	AFB0	0.00 ± 0.00	0.00 ± 0.00	2.30 ± 0.50	<0.001	0.566	<0.001
	AFB2	99.90* ± 5.56	58.80* ± 9.42	38.10* ± 6.40			
AFB1 (ppm)	AFB0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.067	0.035	0.005
	AFB2	0.93^{a} ± 0.27	0.00^{b} ± 0.00	0.00^{b} ± 0.00			
Aeromonas hydrophila challenge							
Mortality rate (%)	AFB0	44.45^{a} ± 2.22	17.78^{b} ± 2.22	11.11^{c} ± 2.22	<0.001	<0.001	0.079
	AFB2	68.89 ^{*a} ± 2.22	37.78^{*b} ±2.22	24.45^{*c} ± 2.22			
Relative percent survival	AFB0	0.00^{c} ± 0.00	58.81^{b} ± 6.47	74.60^{a} ± 5.72	<0.001	<0.001	0.288
	AFB2	-56.35^{*c} ± 3.49	14.29^{*b} ± 8.25	44.44^{*a} ±6.92			

(*) within the same column for each parameter indicates a significant difference between the AFB0 and AFB2 groups. Different superscript letters within the same row indicate a significant difference between GCS levels ($p < 0.05$; n = 3 replicates, 5 fish/replicate). Values are represented as the mean ±SEM. The organ indexes and AFB1 residues were subjected to statistical analysis using the non-parametric Kruskal-Wallis H test.
The experimental groups consisted of two dietary levels of aflatoxin B1: AFB0 (no aflatoxin) and AFB2 (2 mg kg^{-1} diet). Within each level, three levels of GCS were used: GCS0 (0 % GCS), GCS1 (1 % GCS), and GCS2 (2 % GCS).

regulating immunological effector functions through the classical and lectin pathways (Holland and Lambris, 2002). Additionally, Barany et al. (2021a) demonstrated a significant increase in cortisol levels in Gilthead bream after exposure to AFB1. However, fish fed a GCS-supplemented diets exhibited potent immune-stimulatory effect, improved immunological biomarkers, suppressed cortisol levels, and increased resistance to bacterial infection. The rise in white blood cells suggests that GCS enhances immunity, and the improvement in splenic tissue architecture may contribute to the restoration of immune elements. Notably, GC-MS analysis detected several bioactive compounds in GCS, like á-sitosterol (Fraile et al., 2012) and ç-tocopherol (De la Fuente et al., 2008), which have been reported to have beneficial immunomodulatory activity.

The present study revealed clear signs of oxidative stress in fish exposed to AFB1-contaminated feed, as evidenced by decreased antioxidants levels and increased MDA content. Previous research has indicated that AFB1–8,9-epoxide, produced by cytochrome P450, a significant role in aflatoxicosis in fish. This compound can form adducts with cellular macromolecules, including DNA, RNA, and proteins, thereby interfering with their normal functioning (Coppock et al., 2018). Blocking these molecules is an important step in preventing oxidative damage is. Oxidative stress leads to the production of ROS and depletion of antioxidant defense molecules, disrupting cellular redox hemostasis (Aziz et al., 2022). In contrast, groups supplemented with GCS showed an effective antioxidant capability (with higher modulation in GCS2 %), as indicated by increased catalase, SOD, and GPx activities along with decreased MDA levels. GCS have been found to possess high antioxidant capacity and the ability to scavenge various radicals (Zia-Ul-Haq et al., 2012). One of the major phytochemical constituents of GCS, benzene (isothiocyanatomethyl), has been recognized for its potent antioxidant activity (Miyoshi et al., 2004; Nakamura et al., 2004). Furthermore, the GC-MS analyses revealed the presence of 3′, 5′-Dimethoxyacetophenone in GCS, which has prominent pharmacological benefits due to its free radical scavenging activity (Chu and Xiao, 2022). Another bioactive compound detected in GCS, 2-phenylacetonitrile, has been reported to reduce ROS generation and inhibit oxidative stress *in vitro* (Guo et al., 2021).

Herein, the AFB1-contaminated group exhibited significant AFB1 accumulation in the fish muscles, consistent with findings in Nile tilapia by Hassaan et al. (2020). Interestingly, GCS demonstrated a potent antitoxic effect, leading to a noticeable reduction in the AFB1 levels in the AFB1 and GCS treated groups. This reduction may be attributed to the antioxidant capability of GCS (Sanad et al., 2018). It is worth noting that AFB1 residues in the muscle were completely eliminated in the AFB1+GCS treated groups. Previous research by Deng et al. (2010) reported a higher percentage of AFB1 accumulation in the liver of tilapia, with a lower proportion reaching the muscles. Therefore, the recorded improvement of the liver function, architecture, and antioxidant status observed with GCS supplementation in our study may have contributed to the reduction of AFB1 in the liver, consequently inhibiting its deposition in the muscles.

5. Conclusion

This study identifies GCS as a novel and highly effective anti-mycotoxin agent in counteracting the adverse effects associated with AFB1- in *O. niloticus*. The findings suggest that GCS can serve as a growth promoter and antioxidant, and immune stimulator, preserving tissue architecture and mitigate AFB1 residue deposition, which could have positive implications for human health. Therefore, GCS can be considered as a safe and efficient natural supplement for aquafeed. Future studies should explore different GCS levels in other fish species, and the widespread implementation of GCS as an aquafeed supplement in fish farms should be considered.

Ethics statement

The Institutional Animal Care and Use Committee (IACUC) of Zagazig University authorized the experiment (Approval no. ZU-IACUC/2/F/146/2022). The research followed the Ethical Guidelines for the Use and Care of Laboratory Animals in Scientific Investigations published by the National Institutes of Health.

CRediT authorship contribution statement

Walaa El-Houseiny: Conceptualization, Methodology, Visualization, writing—review and editing. **Abd Elhakeem El-Murr:** Conceptualization, Methodology, Resources, data curation, writing—review and editing. **Noura A. Abd-Allah:** Conceptualization, Methodology, Resources, data curation, writing—review and editing. **Yasmina M. Abd-Elhakim:** Conceptualization, Methodology, Resources, Software, Formal analysis, Investigation, Data curation, Visualization, Writing-Original Draft. **Abdel-Wahab A. Abdel-Warith:** Conceptualization, Resources, Software, Formal analysis, project administration, funding acquisition. **Elsayed M. Younis,** Conceptualization, Resources, Software, Formal analysis, project administration, funding acquisition. **Simon J. Davies,** Conceptualization, Resources, Software, Formal analysis, writing—review and editing. **Mohamed M.M. Metwally:** Conceptualization, Methodology, Visualization, writing—review and editing. **Mai E Nasr:** Conceptualization, Methodology, Visualization, writing—review and editing. **Adham A. Al-Sagheer:** Conceptualization, Methodology, Visualization, writing—review and editing. **Bayan A. Hassan:** Conceptualization, Methodology, Visualization, writing—review and editing. **Basma A. Elkhadrawey:** Conceptualization, Resources, Investigation, Data curation, writing—review and editing. All authors contributed to the article and approved the submitted version.

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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102040.

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