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Research Article

Assessment of Various Biochar Sources as a Dietary Additive in *Cirrhinus mrigala* Fingerlings to Enhance Mineral Status, Carcass Composition, and Antioxidant Activity

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MAK conducted the study and wrote the manuscript. SMH administered and supervised the project. SA, AIH and MA analyzed the data. NA, MH and MZHA edited and reviewed the manuscript.

Keywords
Biochar, *Cirrhinus mrigala*, Carcass composition, Antioxidant activity, Mineral status

Abstract | Aquaculture is the fastest industry to produce animal protein but its growth is impeded by the cost of fishmeal, an optimal protein source for fish health. In this study, we examined the feasibility of various biochar supplements with *Moringa oleifera* seed meal (MOSM). Six iso-nitrogenous and iso-energetic diets were prepared: Control (without biochar) and diet II (parthenium biochar), diet III (farmyard manure biochar), diet IV (poultry waste biochar), diet V (vegetable waste biochar) and diet VI (corncob waste biochar). Triplicate groups of 15 fingerlings were kept in V-shaped tanks. In present study, a trial of 90 days was conducted to assess the effects of different types of biochar on body composition, mineral status and antioxidant activity of *Cirrhinus mrigala*. At the conclusion of feeding trial, the protein content was highest (18.51±0.13%) in fingerlings fed with poultry waste biochar and lowest (15.35±0.11%) in fish fed with parthenium biochar. Mineral content was also significantly different in each fish group as compared to control diet. The diet contained Parthenium biochar showed poor results ($P<0.05$) in terms of minerals except Se (0.65±0.004 mg/kg). Antioxidant enzymes activity was the highest ($P<0.05$) in fingerlings fed with poultry waste biochar (peroxidase: 43.48 U/ml, catalase: 46.39 U/ml and superoxide dismutase: 48.20 U/ml) followed by the farmyard manure biochar (peroxidase: 40.77 U/ml, catalase: 43.47 U/ml and superoxide dismutase: 41.19 U/ml). The presented results showed that the different sources of biochars (poultry waste, farmyard manure, vegetable waste and corncob waste biochars), could improve the body minerals, body composition and serum antioxidant status of *C. mrigala* fingerlings except parthenium biochar. This study indicates that biochar could be used as supplement in fish diets to improve mineral status, carcass composition, and antioxidant activity.

Novelty Statement | Our results show efficacy of various sources of biochar on the mineral status, body composition and antioxidant activity of fish which can be further recommended.

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Introduction

Blue foods, also referred to as aquatic foods, produced through aquaculture (Naylor *et al*., 2021), have been a significant part of food used for human consumption (Kakoolaki *et al*., 2013). It is expected that human population will reach over 10 billion so, there is a continuous need for food sources that are high in quality protein (Gentry *et al*., 2017). Fish has become a major nutritional source of animal food due to the growing interest in food's nutritional value. Fish have a high economic and nutritional value, thus there is a growing market for fish and fish products as a more nutrient-dense source of protein (Selamoglu, 2018). Around 3.2 billion individuals worldwide get 20% of their typical animal protein intake from fish. Currently, aquaculture is producing 43% of global seafood supplies and approximately 50% of production is used for human consumption (FAO, 2022). Aquaculture highly depends upon the supply of fishmeal that has traditionally been the protein of choice because of its high nutritional value and capacity to increase feeding activity in aquatic animals (Nunes *et al*., 2014) but it is expensive and not accessible locally (Luhur *et al*., 2021). Fishmeal must be imported in considerable quantities due to the feed industry's rapid growth (Naylor *et al*., 2021). The further use of this finite resource is not sustainable (Naylor *et al*., 2009) because, by 2037, the biological stock limit for feed from wild resources will have occurred (Cottrell *et al*., 2020). Thus, there is a great need of time to produce local alternative sources of protein to replace fishmeal in diets. In this method, transportation costs might be avoided, which would lower costs and perhaps promotes both environmental and economic sustainability. To ease the strain from the use of fishmeal, there has been an increase of interest in employing alternative plant sources in fish feed (Choi *et al*., 2022). Plant-based ingredients are widely utilized in fish diets because they are more readily available and less expensive than fishmeal (Sarfraz *et al*., 2020). The word phytobiotics refers to plant-based feed additives having distinctive phytochemical elements that help increase growth performance, improve immunity (Abdel-Latif *et al*., 2020) and they also promote health of fish (Hamed *et al*., 2021). The presence of essential amino acids, carotenoids in the leaves, and other elements with nutraceutical attributes support the idea of using *Moringa oleifera* as a nutritional supplement in feed preparation (Abdull Razis *et al*., 2014). Most of the tropics and subtropics have grown *M. oleifera,* which has a wide range of uses and is extremely significant economically (Abbas, 2013) and it also has fast growth (Falowo *et al*., 2018). Due to its health advantages, it is also known as the miracle tree, drumstick tree, and tree of life. It has antimicrobial, anti-inflammatory, antioxidant and hepatoprotective properties so that's why its use has been encouraged as feed additive in aquafeeds. Fish and livestock can eat the leaves, roots, bark, seeds, flowers, and pods of the moringa tree (Kou *et al*., 2018).

Incomplete pyrolysis of different organic materials like wood, manure, straw and leaves produces biochar under given conditions of approximately 550 ˚C and limited oxygen (Lehmaan and Joseph, 2015). Biochar is derived from charcoal using some processes and activated charcoal is its precursor (Azargohar and Dalai, 2006). Over the past ten years, researchers have examined the effects of incorporating biochar into animal feed for ruminants, pigs, and fish (Preston, 2014; Schmidt *et al*., 2017). It can boost the growth and survival rates of fish (Mabe *et al*., 2018). Past researches showed that biochar could be a great feed ingredient that might enhance animal growth, blood profiles, boost immunity, and decrease infections. Untreated, natural trunk wood, which is 100 percent biodegradable, ought to be used as the raw material for biochar products, used as feed supplements (Man *et al*., 2021). In 21st century, application of biochar has become the multidisciplinary research topic due to its special characteristics (Chen *et al*., 2019). Thu *et al*. (2010) reported that feeding Japanese flounder with 0.5% bamboo wood charcoal improved the content of crude protein via improving protein digestion. Moreover, the growth performance, enzyme activities (lipase and amylase) and hematology were also increased by feeding biochar to fish (Kara *et al*., 2023).

The detritus-eating major carp, Cirrhinus mrigala, is an essential part of the polyculture with the other species of major carps. The Ganges and Indus River basins are home to the *C. mrigala* or mrigal (subfamily: Cyprininae, family: Cyprinidae) in its natural habitat. The species is naturally found throughout Bangladesh, Nepal, India, and Pakistan. Due to its high consumer preference and potential for aquaculture, the species is important commercially (Chauhan *et al*., 2007). The aim of this study was to determine the effects of employing various biochar sources to enhance the mineral status, body composition, and antioxidant enzyme activity in *C. mrigala* fingerlings.

Materials and Methods

Experimental conditions

From the Government Fish Seed Hatchery in Faisalabad, fingerlings of *C. mrigala* were bought, and they were kept in V-shaped containers that were made specifically for collecting faeces. For two weeks, fingerlings were acclimatized to the experimental environment. Fingerlings were immersed in NaCl (5%) prior to the start of the feeding trial for making sure that fingerlings have no ecto-parasites present in them. Each tank had 15 fingerlings, which were kept there for a total of 90 days.

Biochar production

After crushing and drying, several forms of biomass, including as parthenium, poultry waste, vegetable waste, corncob waste, and farmyard manure, were collected and used for biochar production. Biochars were pyrolyzed individually in a top lit-up-draft gasifier (TLUD) at 500°C for 90 minutes. Following that, they were cooled and passed through a sieve (2 mm) to be crushed into tiny particles (Tripathi *et al*., 2016). The powder of each biochar was then kept in a sealed container until it was used in the preparation of experimental diets.

Formulation of experimental diets

The diets were formulated with careful consideration of the requisite nutrient amounts necessary for the optimal growth of fish, as recommended by AOAC (2005). All components were procured from a commercial feed mill and subjected to chemical analysis. MOSM (52%) served as the foundational diet component, as outlined in Table 1. Additionally, an inert marker, specifically Cr_2O_3, was incorporated into the recommended diets at a 1% inclusion rate. A total of six diets were formulated. The initial diet served as the control, containing no biochar, while the subsequent five diets were differentiated by the inclusion of various types of biochar: Parthenium (group II), farmyard manure (group III), poultry waste (group IV), vegetable waste (group V), and corncob waste biochar (group VI). The feed components were finely milled to pass through a 0.5 mm sieve. These components were then blended in a mixer for five minutes, during which fish oil was incrementally added. The mixture required the addition of 10% to 15% water to achieve the appropriate dough consistency, as noted by Lovell (1989). Subsequently, feed pellets were produced using a pelleting machine.

Procedure of feeding and collection of feces

C. mrigala fingerlings were given the prescribed feed at a rate of 5% of live wet weight twice each day (AOAC, 2005). Three replicates for each experimental diet have been assigned. After the 120-minute feeding time, the valves in each tank were opened to let the remaining diet escape. The additional diet was completely cleaned out of the tanks before they were replaced with water. The estimated nutritional content of the faeces was determined after they had been collected and dried in an oven.

Carcass analysis

At the end of experimental period, three fish were chosen at random from each tank, and their complete bodies were chemically analyzed. Mortar and pestle was used for homogenization of fish samples. Chemical analyzation of samples was done according to standard methods of AOAC (2005). By drying in an oven at 105°C for 12 h, then the moisture content was determined, while the micro Kjeldahl apparatus was used to calculate the amount of crude protein (CP). Soxtec HT2 1045 system was used to extract ether extract (EE) by following petroleum ether extraction protocol whereas ash was found out by combustion in electric furnace for 12 h at 650 °C.

Analysis of mineral content

A mixture of boiling nitric acid and perchloric acid (2:1) was used to digest individual whole-body samples (AOAC, 2005). Mineral contents were determined by atomic absorption spectrophotometer after adequate dilution. Using a calorimeter (UV/VIS spectrophotometer, YR01862, Kalstein), the phosphorus was measured at 350 nm.

Table 1: Composition of ingredients in experimental diets (%).

Ingredients	Control	Parthenium	Farmyard manure	Poultry waste	Vegetable waste	Corncob waste	
Biochar (g/kg)	0	2	2	2	2	2	
MOSM*	52	52	52	52	52	52	
Fish meal	16	16	16	16	16	16	
Rice Polish	9	9	9	9	9	9	
Fish oil	7	7	7	7	7	7	
Wheat flour	12	10	10	10	10	10	
Chromic oxide	1	1	1	1	1	1	
Vitamin premix**	1	1	1	1	1	1	
Ascorbic acid	1	1	1	1	1	1	
Mineral premix***	1	1	1	1	1	1	
Proximate composition (%)							
	Dry matter	**Gross energy**	**Crude protein**	**Carbohydrates**	**Crude fibre**	**Crude fat**	**Ash**
MOSM*	91.45	3.46	34.78	43.85	2.31	9.08	10.43
Fish meal	90.77	3.05	54.56	19.23	1.12	6.43	23.16
Rice Polish	92.67	3.31	12.32	51.53	10.28	13.97	11.18
Wheat flour	92.01	3.12	9.22	82.96	2.24	2.12	1.97

* MOSM: *M. oleifera* seed meal. **Vitamin (Vit.) premix /kg: Vit. A: 15,000,000 IU, Vit. B6: 4000 mg, Vit. K3: 8000 mg, Vit. C: 15,000 mg, Vit. B12: 40 mg, Vit. D3: 3,000,000 IU, B2: 7000 mg, Ca pantothenate: 12,000 mg, Nicotinic acid: 60,000 mg, Folic acid: 1500 mg. ***Mineral premix /kg: Mn: 2000 mg, Zn:3000mg, Fe: 1000 mg, Cu: 600 mg, Ca: 155 g, P: 135 g, Mg: 55 g, Na: 45 g, Co: 40 mg, I: 40 mg, Se: 3 mg.

Table 2: Mineral status in *C. mrigala* fingerlings fed different types of biochar supplemented MOSM based test diets.

Minerals	Test diet-I	Test diet-II	Test diet-III	Test diet-IV	Test diet-V	Test diet-VI	P-value
	Control diet	Biochar types					
		Parthenium	Farmyard manure	Poultry waste	Vegetable waste	Corncob waste	
Ca (%)	0.76±0.04^{cd}	0.70±0.01^{d}	0.94±0.02^{ab}	1.07±0.03^{a}	0.87±0.05^{bc}	0.80±0.06^{bcd}	0.0000 ***
Na (mg/g)	4.20±0.03^{e}	3.84±0.07^{f}	5.33±0.04^{b}	5.56±0.01^{a}	4.90±0.03^{c}	4.61±0.05^{d}	0.0000 ***
K (%)	6.84±0.04^{e}	6.45±0.03^{f}	7.92±0.02^{b}	8.45±0.06^{a}	7.36±0.01^{c}	7.05±0.07^{d}	0.0000 ***
P (%)	1.34±0.02^{bc}	1.28±0.04^{c}	1.59±0.05^{a}	1.65±0.01^{a}	1.50±0.03^{ab}	1.42±0.06^{bc}	0.0000 ***
Fe (ug/g)	44.25±0.24^{e}	42.24±0.30^{f}	50.84±0.14^{b}	52.69±0.39^{a}	48.65±0.46^{c}	45.2±0.29^{d}	0.0000 ***
Cu (ug/g)	2.70±0.05^{d}	2.23±0.06^{e}	4.04±0.07^{b}	4.72±0.02^{a}	3.95±0.06^{b}	3.40±0.07^{c}	0.0000 ***
Mg (%)	2.90±0.03^{e}	2.55±0.04^{f}	3.51±0.05^{b}	3.70±0.06^{a}	3.33±0.03^{c}	3.12±0.02^{d}	0.0000 ***
Zn (ug/g)	2.81±0.01^{e}	2.22±0.05^{f}	4.04±0.03^{b}	4.65±0.02^{a}	3.84±0.06^{c}	3.11±0.04^{d}	0.0000 ***
Mn (ug/g)	8.74±0.12^{e}	7.32±0.037^{f}	10.12±0.49^{b}	11.82±0.21^{a}	9.94±0.18^{c}	9.01±0.40^{d}	0.0000 ***
Se (mg/g)	0.54±0.01^{ab}	0.65±0.004^{a}	0.29±0.02^{d}	0.15±0.05^{e}	0.39±0.04^{cd}	0.43±0.06^{bc}	0.0000 ***

At ($p<0.05$), the means of rows with different superscripts vary substantially. The data are the mean of three replicates.

Evaluation of antioxidant activity of Enzymes

Three fish from each tank were taken for blood samples. They were anesthetized with a 150 mg^{-1} tricane methane sulfonate solution and blood was drawn from the caudal vein. The samples were immediately centrifuged at 3,000 g for 10 minutes at 4°C. The serum was collected and kept at 70°C for the measurement of antioxidant parameters (Feng *et al.*, 2014). Using a commercial kit (Cayman's Assay Kit, Cayman Chemical, Ann Arbour, Michigan), the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels were assessed in the plasma samples.

Statistical analysis

The data of body composition, antioxidant enzymes and minerals were calculated using One-way Analysis of Variance (ANOVA) (Steel *et al.*, 1996). To separate means, Tukey's Honesty significant difference test was employed, with a significant level of $P<0.05$ (Snedecor and Cochran, 1991). Version 6.303 of the Costat Computer Package (PMB 320, Monterey, CA, 93940USA) was utilized for statistical analysis.

Results and Discussion

Mineral status

Table 2 displays the mineral analysis of *C. mrigala* fingerlings. The mineral contents of each group varied significantly ($P<0.05$), according to the results. The fingerlings fed on MOSM based diet supplemented with poultry waste biochar had the highest ($P<0.05$) amounts of Ca, Na, P, Mg, Fe, Mn, Zn, K, and Cu (1.07%, 5.56 mg/g, 1.65%, 3.70%, 52.69 ug/g, 11.82 ug/g, 4.65 ug/g, 8.45%, and 4.72 ug/g, respectively). The fish fed test diets III (farmyard manure), IV (poultry waste), V (vegetable waste) and VI (corncob waste) showed significantly different outcomes ($P<0.05$) as compared to control (test diet I). The fingerlings given parthenium biochar (test diet II) showed the lowest amounts of Ca, Na, P, Mg, Fe, Mn, Zn, K, and Cu (0.70%, 3.84 mg/g, 1.28%, 2.55%, 42.24 ug/g, 7.32 ug/g, 2.22 ug/g, 6.45%, and 2.23 ug/g, respectively). The test diet IV yielded maximum levels for all minerals, with the exception of Se. While Se content was highest (0.65 mg/g) in fingerlings fed test diet II.

Carcass composition

Figures 1 and 2 depict the carcass composition results of *C. mrigala* fed with various test diets incorporating biochar. The findings of the carcass composition analysis revealed that fingerlings fed test diet IV had the lowest amount of ash (1.97%) and the highest content of protein (18.51%), followed by test diet III that had the second-highest protein (18.14%) and second-lowest ash (1.99%) values. Conversely, fish fed test diet II had the highest percentage of ash (2.52%) and the lowest value of protein (15.35%). Test diet IV had the lowest percentages of fat (4.36%) and moisture (75.16%).

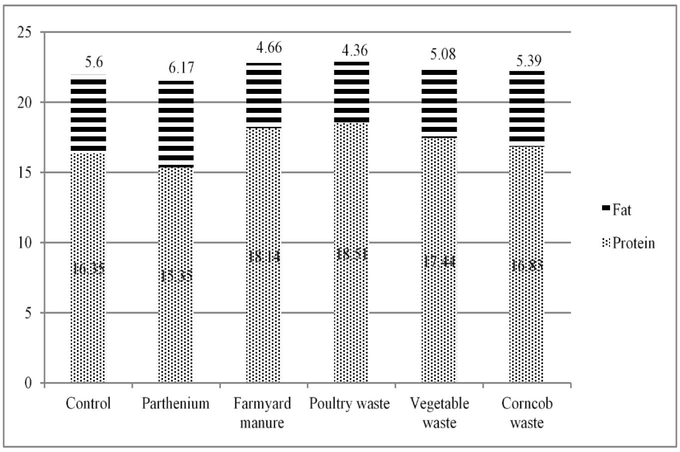

Figure 1: Protein and fat content of *C. mrigala* fingerlings fed on different types of biochar supplemented with MOSM based test diets.

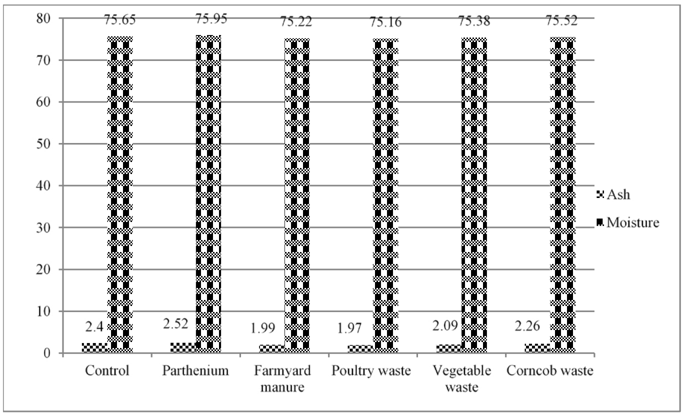

Figure 2: Ash and moisture content of *C. mrigala* fingerlings fed on different types of biochar supplemented with MOSM based test diets.

Antioxidant activity

Table 3 displayed the SOD, GPx and CAT activities in *C. mrigala* showing highest amount of these antioxidant enzymes in fish fed with MOSM based diet supplemented with poultry waste biochar (GPx: 43.48 U/ml, CAT: 46.39 U/ml and SOD: 48.20 U/ml). Fingerlings fed with control diet (GPx: 32.62 U/ml, CAT: 34.87 U/ml and SOD: 29.52 U/ml) and diet containing parthenium biochar showed the lowest results (GPx: 28.37 U/ml, CAT: 27.59 U/ml and SOD: 24.61 U/ml).

Aquaculture industry is highly being dependent upon the supply of an ideal feed which can fulfill the nutritional requirement of fish therefore fishmeal has been extensively used as feed for both carnivorous and omnivorous species. Many feed formulations used in aquaculture contain 50% of fishmeal until now (Selamoglu, 2018; Macusi *et al*., 2023) which causes it to become expensive. That's why there is a need to replace fishmeal with good protein sources without having negative impacts on fish health. In recent study, different types of biochar were used to study their effects on fish health (Kara *et al*., 2023). Many studies have used biochar generated from waste and biomass materials to remediate aquaculture effluent (Reda *et al*., 2022); however, few studies have looked into using biochar as a feed supplement in various animal species (Schmidt *et al*., 2017). Since biochar improves the health of organisms, it will eventually become a well-known supplement in future (Cheng *et al*., 2023).

The carbon content of charcoal, which ranges from 85 to 95%, its large surface area, and its pores, all help fish grow, absorb nutrients more easily, and are more feed-efficient (Thaib *et al*., 2021). Charcoal has been used as a feed additive for many terrestrial animals because of its ability to absorb gases, especially nitrogen and ammonia, stimulate digestive function, and eliminate poisons and pollutants from an animal's gastrointestinal tract (Mekbungwan *et al*., 2004). In our study, poultry waste biochar showed the best results in terms of whole-body composition. In another study, fish fed with activated charcoal showed enhanced CP ratio and also higher absorption and digestion of nutrients (Abdel-Tawwab *et al*., 2017) but the mechanism of charcoal digestion in fish has not been studied or explained yet. This study also exhibited that charcoal supplementation improved lipid metabolism in *Oreochromis niloticus*. According to Thu *et al*. (2010), supplementing with bamboo wood charcoal increased the content of CP by enhancing protein digestion; however, there was no discernible change in moisture, ash or fat content. Typically, variations in their rates of synthesis and/or deposition in fish bodies could be connected to variations in their protein and lipid levels (Abdel-Tawwab *et al*., 2006). In a study, digestion was improved by addition of biochar in striped catfish feed which could be due to the fact that it provides the habitat for gut microbiota which could make them more efficient (Lan *et al*., 2016).

Charcoal consists of many minerals like zinc, calcium, potassium, iron, copper, manganese etc which could improve the health of fish and it can also absorb toxic gases which protect fish from water pollution (Abdel-Tawwab *et al*., 2017). In our study, poultry waste biochar showed the best results in terms of mineral status. By improving the mineral intake of the hens through the addition of wood charcoal to their food, which contains high levels of magnesium, calcium, potassium, and other minerals, broken egg incidence can be decreased. In addition, wood charcoal is an adsorbent that can condition cell membranes, lower surface tension by getting rid of gases, toxins, or other unpleasant substances in the gastrointestinal system,

Table 3: Antioxidant activity of peroxidase, catalase and superoxidase dismutase of *C. mrigala* fed different types of biochar supplemented MOSM based test diets.

Antioxidant enzymes	Test diet-I	Test diet-II	Test diet-III	Test diet-IV	Test diet-V	Test diet-VI	P-value
	Control diet	Biochar types					
		Parthenium	Farmyard manure	Poultry waste	Vegetable waste	Corncob waste	
Peroxidase (U/mL)	32.62±0.30^{e}	28.37±0.19^{f}	40.77±0.16^{b}	43.48±0.10^{a}	36.68±0.64^{c}	34.56±0.28^{d}	0.0000***
Catalase (U/mL)	34.87±0.09^{e}	27.59±0.29^{f}	43.47±0.36^{b}	46.39±0.47^{a}	41.22±0.35^{c}	36.47±0.29^{d}	0.0000***
Superoxide dismutase (U/mL)	29.52±0.16^{e}	24.61±0.25^{f}	41.19±0.41^{b}	48.20±0.45^{a}	37.40±0.20^{c}	33.40±0.24^{d}	0.0000***

At ($p < 0.05$), the means of rows with different superscripts vary substantially. The data are the mean of three replicates.

and enhance nutrient absorption and utilization across the membranes (Kutlu *et al*., 2001). Poor results shown by parthemium biochar could be attributed to the fact that addition of black colored charcoal could decrease the palatability of feed (Jindal *et al*., 1994). The kind of fish species, size, biochar types, and other variables alter the outcomes of study (Duran and Talas, 2009).

In recent study, antioxidant enzymes have been studied to assess the fish health status. In order to generate and detoxify free radicals, fish have both enzymatic (mitochondrial and cytosolic SOD), non-enzymatic (reduced GPx and vitamin E), and CAT antioxidants (Lushchak, 2011). As indicators of pollutant-induced oxidative stress in aquatic species, the enzymes SOD (which detoxifies harmful superoxide anion radicals) and CAT (primary antioxidant defense component) can be employed (Borković *et al*., 2008). According to Kara *et al*. (2023), when genetically modified farmed tilapia (GIFT) was fed with sugarcane bagasse biochar, enzymatic activities (lipase and amylase) were enhanced. The redox state of fish cells depends on these antioxidants. Similarly, changes in the concentration of lipid peroxidation are also utilized as a biomarker of effect and to reflect severe oxidative damage (Van der Oost *et al*., 2003). Our study has illustrated that parthenium biochar is not a viable option for supplementation in fish feeds, as it led to a reduction in the activity of antioxidant enzymes.

Conclusions and Recommendations

The findings from the recent investigation indicated that biochar can be included as an additive in the feed of *C. mrigala*, as compared to the control group. All sources of biochars lead to notable positive alterations in enzymatic activities, mineral content, and body composition of the fish, with the exception of parthenium biochar. To enhance our understanding of the physiological significance of biochar, additional research is warranted to evaluate the influence of different biochar types on fish health.

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Data availability statement

Data will be available on request.

Conflicts of interest

The authors have declared no conflict of interest.

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