Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 27(3): 231 – 250 (2023)
www.ejabf.journals.ekb.eg

Immunostimulatory effect of macroalga *Padina pavonica* on the performance of the Nile tilapia, *Oreochromis niloticus* challenged with *Aeromonus hydrophila*

Mai Nashaat[1*], Samar S. Marzouk[1], Heba M. Ezz El-Din[2], Asmaa Maghawri [3]

[1] National Institute of Oceanography and Fisheries, Aquaculture Division, (NIOF), Egypt
[2] National Institute of Oceanography and Fisheries, Marine Environment Division, (NIOF), Egypt
[3] Department of Aquaculture Diseases Control, Fish Farming and Technology Institute (FFTI), Suez Canal University, 41522 Ismailia, Egypt

***Corresponding author: mainashat85@gmail.com**

ARTICLE INFO

Article History:
Received: May 8, 2023
Accepted: May 26, 2023
Online: May 30, 2023

Keywords:
Aeromonus hydrophila,
Body composition,
Brown alga,
Tilapia,
Oxidative enzymes,
Polysaccharides

ABSTRACT

Immunostimulation by natural products, such as brown algae presents an alternative choice to antibiotics and vaccines in aquaculture. The present study was performed to determine the effects of dietary brown alga *Padina pavonica* on the body composition measurements, antioxidant activity and innate immunity of *Oreochromis niloticus* before and after being challenged with *Aeromonus hydrophila*. The Nile tilapia (4.58 ± 0.03 g) samples were fed commercial pellets mixed with four concentrations of *P. pavonica* (2.0, 4.0, 6.0 and 8.0g/ kg) for 45 days under two different conditions: experimental (A) and farming (B). The results indicate that fish fed higher algal dose T4 (8.0g/kg) witnessed an improved growth. All immunological parameters, including oxidative enzymes, somatic indices and lysozyme activities were assessed for both environments (A and B). The histological structure of the liver, spleen and intestine of fish showed some enhancements in treated group cells relative to the control fish. All groups after the feeding trials were challenged with pathogenic bacteria *Aeromonus hydrophila*. No changes were detected in oxidative enzyme activities for both environments, while lysozyme activities recorded a reverse effect upon decreasing the higher algal dose subgroups T4. In addition, a decrease was recorded in the mortality rate in both environments after feeding with *P. pavonica* alga in all subgroups (10 and 20%), which indicates the defense effect of brown alga *P. pavonica* against the pathogenic bacteria.

INTRODUCTION

Aquaculture is nowadays considered as a main source of protein with lower cost compared with other animal protein sources **(Dawood *et al.*, 2021)**. Numerous studies declared that the immunostimulation using natural products extracted from plants is highly recommended **(Kosanić *et al.*, 2015; Wang *et al.*, 2015)**. Recently, a growing interest has emerged as a global trend towards the usage of seaweeds in fish diets; it has positive influences on the fish immune system, as well as producing biologically active secondary metabolites; these impacts have been widely documented **(Zaim *et al.*, 2020)**.

Brown seaweeds have a large amount of alginic acid and fucoidan, which are polysaccharides. These ingredients have positive influences on fish immune system. Macroalga *Padina* sp. is rich with polysaccharides, carbohydrates, proteins, lipids, fatty acids, ashes and dietary fibers **(Subramanian *et al.*, 2015)**. *P. pavonica* has recently gained the attention of researchers for its importance as a natural source of immunostimulant, antibiotic, antioxidant, antimicrobial, hepatoprotective, hypo-allergenic, anti-inflammatory, antidiabetic and as a good source of food and bio-fertilizer **(Ansari *et al.*, 2019)**.

The study aimed to evaluate the role of the brown alga *P. pavonica* on the body composition, lysozyme, and antioxidant enzymes activity of *Oreochromis niloticus* before and after challenged with *Aeromonus hydrophila*. In addition, the histopathological effect on some immunological organs was addressed and the structure analysis of polysaccharides in *P. pavonica* was performed using Infrared (IR) spectroscopy.

MATERIALS AND METHODS

1. Ethical statement

All the experimental protocols including animals were carried out according to the guidelines of the Ethics of Animal Use in the Research Committee (EAURC), Faculty of Science, Suez Canal University, Egypt, with approval number (REC40/2022).

2. Collection, preparation and identification of polysaccharides from alga

Fresh brown *Padina pavonica* was obtained from the coastal water in Marsa Allam, Red Sea in the summer of 2020. Based on its morphology, the taxonomic reference was used for identification as mentioned by **Sahoo *et al.* (2002)**. Dehydration and preparation of powder alga was done according to **Gamal-Eldeen *et al.* (2009)**.

3. Fourier-transform infrared (FT-IR) spectroscopy analysis

The polysaccharides extracted from *P. pavonica* were prepared in thin pellets using potassium bromide (KBr) for FTIR analysis. IR spectra were obtained from PERKIN ELMER model at the resolution of 1 cm^{-1} in the range of 3500 to 500 cm^{-1}.

4. Experimental fish

A total of 390 apparently healthy cultured *O. niloticus*, with average body weight of 4.54 ± 0.03 g were obtained from the Sustainable Development Center of Fish Farming and Technology Institute (FFTI), Suez Canal University, Egypt. They were acclimated for two weeks in fully prepared 10 fiberglass tanks, and then divided into two main

groups, where group (A) was cultured in tanks filled with dechlorinated tap water (experimental condition), and group (B**)** was cultured in tanks of freshwater (farming condition); each group included 195 fish. Each group was further subdivided into five subgroups, each of which had three replicates (13 fish per tank) reared in a 50-liter glass tank. Fishes were held under natural photoperiod condition throughout the experimental period (45 days) feeding trials and (15 days) challenge test (Table 1).

Table 1. Water parameters in environments (A) and (B)

Parameter	Experimental environment (A)	Farming environment (B)
Capacity (L)	500	500
Fish number	195 (13/tank)	195 (13/tank)
DO (mg/L)	6.25–8.3	6.4–8.5
T (°C)	23–24	22–24
pH	7.90–8.2	7.78–8.11
Ammonia (TAN*)	0.13–0.15	0.12–0.14

DO: Dissolved oxygen (mg/L); T: Temperature (°C); * TAN: Total ammonia nitrogen.
The control subgroups (CA/CB), means feeding commercial pellets without additives, (T) indicates for treatments of *P. pavonica* algae as: T1A/T1B: 2.0 g/kg, T2A/T2B: 4.0 g/kg, T3A/T3B:6.0 g/kg and T4A/T4B:8.0 g/kg.

5. Diet preparations, culture conditions and feeding trials likewise (Maghawri *et al.*, 2023)

6. Fish body composition in environments (A) and (B)

The whole body composition of the Nile tilapia was determined for three fish from each subgroup in both environments by using the standard methods mentioned the study of **Folch *et al.* (1957)** and set by the Association of Official Analytical Chemists **(AOAC, 1997)**.

7. Hepatosomatic, spleenosomatic and gonadosomatic indices (HIS, SSI and GSI)

Fishes were dissected and the internal organs of liver, spleen and gonads were dissected. HIS, SSI and GSI were calculated using the following formulas of **Pandit and Gupta (2019)**:
HSI= weight liver (g.)/weight of fish (g.) ×100, SSI= weight of spleen (g.)/weight of fish (g.) ×100, GSI =weight of gonads (g.)/ weight of fish (g.) ×100.

8. Histopathological examinations

Liver, spleen, and intestine samples were preserved in 10% neutral formalin for 24 hours before being transferred to 70% ethanol for complete preservation. The specimens were prepared and stained with hematoxylin and eosin stain (H & E) according to **Drury and Wallington (1987)** and **Bancroft *et al.* (1996)**.

9. *Aeromonus hydrophila* preparation and injection

Aeromonus hydrophila strain was supplied by the Central Lab, Faculty of Veterinary Medicine, Suez Canal University, Egypt. The bacteria were diluted in phosphate-buffered saline (PBS, pH 7.2) and adjusted to a concentration of 2×10^7 CFU/ml. After finishing the 45 days feeding trials, ten fish per subgroups were intraperitoneally (IP) injected with 0.2 mL/fish of (2.6×10^6 CFU) *A. hydrophila* **(Ran *et al.*, 2018)**. Fish were continuously fed for 15 days after injection, and the clinical signs and mortalities were recorded **(Saputra *et al.*, 2016)**.

10. Evaluation of oxidative biomarkers and antioxidant enzymes activity before and after challenged with *A. hydrophila* in environments (A) and (B)

The activities of antioxidant enzymes were assessed in liver before and after challenge with *A. hydrophila*. Homogenization of 0.5g of liver tissue was performed from all subgroups. The tissues were preserved frozen at -20^{O}C till processing. The reagents were purchased from Bio-diagnostic company (Diagnostic and research agency). Malondialdehyde (MDA, CAT no. 25. 29) was measured according to previous studies **(Ohkawa *et al.*, 1979; Del Rio *et al.*, 2003)**. Superoxide dismutase (SOD; SD 25.21) activity was assessed according to **Paoletti *et al.* (1986)**. Reduced glutathione (GSH) was evaluated using reduced glutathione Assay Kit (GSH, CAT no. GR 25.11). Glutathione peroxidase (GPx; CAT no. GP 25.24) activity was determined according to **Beutler (1971)**. Catalase (CAT; CAT no. CA 25.17) activity was assessed according to the method of **Beers and Sizer (1952)**.

11. Lysozyme activity before and after challenged with *A. hydrophila* in environments (A) and (B)

Lysozyme activities were assessed in serum from all subgroups before and after *A. hydrophila* infection according to the methods described in the study of **Ellis (1990)**.

12. Statistical analysis

Results were presented as means ± the standard error, and they were assessed by one-way analysis of variance (ANOVA) using IBM SPSS statistics 20.0. Duncan's multiple-range test and descriptions were used to ascertain differences among treatments, with significance set at $P \leq 0.05$ according to **Dytham (2011)**.

RESULTS

1. ***Padina pavonica* effective component**

FT-IR spectrum contains band at 3278 cm^{-1} falls within the intensity of signals detected for compounds with (-OH) stretching. Two peaks at 1614-1411 cm^{-1} are originated from the asymmetric stretching vibrations of the carboxylate (COO^-). An intense peak centering at 1024 cm^{-1} was attributed to the presence of

stretching vibrations of the glycosidic linkage formation in polysaccharide (C─O─C). Moreover, medium sharp absorption band at 868 cm^{-1} shows the C─O─S bending vibration and confirms the presence of a sulfate group. Furthermore, weak absorptions region observed below band 789 cm^{-1} are related to the $(CH_2O)_2$ carbohydrate groups, (Fig. 1 & Table 2).

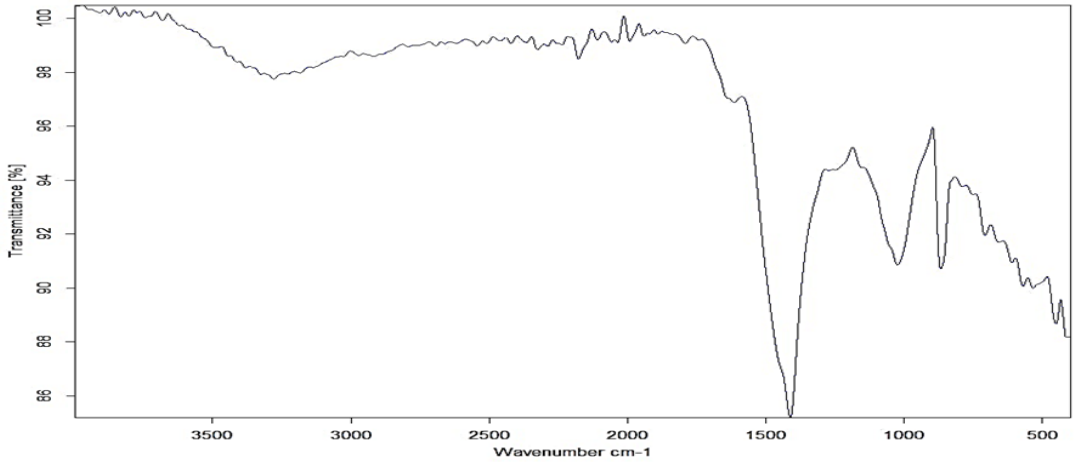

Fig. 1. FTIR spectra for polysaccharides of *P. pavonica*

Table 2. A list of infrared (IR) vibrational modes characteristic to some polysaccharides is summarized at wave number (cm^{-1}) **(Pereira *et al.*, 2013)**

IR absorption wave number (cm^{-1})	Signal characteristics
3500-3200	The broad peak signifies the stretching vibrations of the OH group
1650	Represent the carbonyl group of a carboxylic acid group
1135	Stretching vibrations of the glycosidic C─O bond
1315-1220 and 1140-1050	Symmetric and asymmetric stretching vibrations of the RO─SO3- bond of the sulfate groups
1370	Sulfate groups S═O
1250	The asymmetric stretching of S═O
930	The vibration of the C─O─C bridge of 3,6-anhydro-L-galactose and 3,6-anhydro-D-galactose (common to both agar and carrageenan)
890	Anomeric CH of β-galactopyranosyl residues
840	Sulfation on C4 galactose
830	Sulfation on C2 galactose units
805	Sulfation on C2 of the 3,6-anhydro-L-galactose
740 and 716	C─O─C bending vibrations in glycosidic linkages
1210-1280	Broadband represents the sulfate group
822	Mannuronic unit (characteristic band)
808	Guluronic unit (characteristic band)

2. Body composition

As shown in Table (3), a highly significant difference (*P≤0.05*) was detected between subgroups. In experimental environment (A), moisture, protein and ash were significantly increased in T4 subgroup, while lipid recorded a significant increase in T3. In farming environment (B), moisture and protein were significantly increased in T4 subgroup, while ash was significantly increased in T1. While, lipid was significantly increased in T2.

Table 3. Effect of dietary *P. pavonica* levels on whole body composition (%wet weight basis) of *O. niloticus* in environments (A) and (B)

Subgroups / Factor		*Padina pavonica* (g/kg diet)					*P. Value*
		(0) Control	2.0 (T1)	4.0 (T2)	6.0 (T3)	8.0 (T4)	
Moisture	A	71.79±0.10 ^{b}	74.55±0.46^{a}	68.92±0.72 ^{c}	70.82±0.23 ^{b}	73.95±0.39 ^{a}	0.00
	B	72.19±0.25 ^{b}	72.55±0.29 ^{b}	69.15±0.34 ^{c}	73.54±0.43 ^{a}	74.21±0.25 ^{a}	0.00
Protein	A	14.97±0.17 ^{b}	11.96±0.03 ^{c}	10.07±0.41 ^{d}	9.02±0.31 ^{e}	16.69±0.42^{a}	0.00
	B	11.53±0.12 ^{b}	12.20±0.13 ^{b}	8.52±0.89 ^{c}	14.56±0.23 ^{a}	14.68±0.10 ^{a}	0.00
Ash	A	3.53±0.38 ^{b}	5.56±0.44 ^{a}	3.33±0.32 ^{b}	6.34±0.06 ^{a}	5.99±0.09 ^{a}	0.00
	B	5.57±0.19 ^{b}	6.16±0.16 ^{a}	4.33±0.29 ^{c}	5.17±0.14 ^{b}	3.58±0.18 ^{d}	0.00
Lipid	A	7.64±0.15 ^{bc}	8.86±0.49 ^{b}	6.29±0.91 ^{c}	11.35±0.12 ^{a}	7.89±0.29 ^{b}	0.00
	B	7.99±0.41 ^{c}	6.39±0.29 ^{d}	10.73±0.25 ^{a}	9.69±0.32 ^{b}	5.63±0.11 ^{d}	0.00

Note that: a-d means there was statistically significant difference between values at $P \leq 0.05$ within the same row. (n=10); one way ANOVA test was used to compare means.

3. Hepatosomatic (HSI), spleenosomatic (SSI), and gonadosomatic (GSI) indices in environments (A) and (B)

From Table (4), GSI recorded a highly significant difference between subgroups ($P \leq 0.05$) in both environmental conditions (A) and (B). T4 was significantly increased in both environments, (A) and (B). HIS recorded a highly significant difference within groups ($P \leq 0.05$) in experimental environment (A), with the maximum value in T3, while in farming environment (B), the values recorded no significant difference within subgroups ($P > 0.05$). SSI values recorded no significant difference between subgroups ($P > 0.05$) among experimental condition subgroups (A); while in farming conditions (B), a highly significant difference was detected among subgroups ($P \leq 0.05$), with maximum values in T4 subgroup.

Table 4. Hepatosomatic (HSI), spleenosomatic (SSI) and gonadosomatic (GSI) indexes in environments (A) and (B)

Subgroups / Factor		*Padina pavonica* (g/kg diet)					*P. Value*
		(0) Control	2.0 (T1)	4.0 (T2)	6.0 (T3)	8.0 (T4)	
GSI	A	1.59±0.16^{c}	1.19±0.26^{c}	2.84±0.39^{b}	2.08±0.29^{bc}	3.94±0.35^{a}	0.00
	B	2.23±0.17^{d}	3.83±0.47^{bc}	4.92±0.41^{ab}	2.65±0.23^{cd}	5.61±0.68^{a}	0.00
HSI	A	3.09±0.26^{b}	2.76±0.26^{b}	3.24±0.60^{b}	4.75±0.50^{a}	2.13±0.26^{b}	0.00
	B	2.82±0.41^{a}	3.01±0.23^{a}	3.13±0.29^{a}	3.01±0.24^{a}	2.54±0.29^{a}	0.67
SSI	A	0.34±0.05^{a}	0.28±0.04^{a}	0.32±0.03^{a}	0.38±0.04^{a}	0.29±0.04^{a}	0.43
	B	0.29±0.05^{b}	0.32±0.04^{b}	0.16±0.02^{b}	0.15±0.02^{b}	1.26±0.37^{a}	0.00

Note that: a-d means there was statistically significant difference between values *at (P*≤ 0.05) within the same row. (n=10), one way ANOVA test to compare means

4. Histological and histopathological findings in the experimental and farming environments (Groups A and B)

4.1. Intestine

The results of histological sections for the control subgroup (CA) showed that the intestine had irregular villi with edema and chronic inflammations in the lamina propria in the control sample, whereas the intestine showed regular villi with no edema or chronic inflammations. Subgroup T1A showed uniform intestinal villi and crypts, with mild chronic inflammation in the lamina propria. While subgroup T3A showed normal intestinal villi with no sign of inflammation. On the other hand, T4A showed regular villi, with no inflammation in the lamina propria. The intestine of farming subgroup (B) showed that the control subgroup (CB) section showed edema in villi cores and lamina propria with chronic inflammations, whereas the subgroup T1B section showed irregular villi with no atrophy or inflammations. T2B showed regular villi with no atrophy. T3B showed uniform intestinal villi and crypts with mild chronic inflammation in the lamina propria, while subgroup T4B showed regular villi with no inflammation in the lamina propria (Fig. 2a- j).

4.2. Liver

Liver of the control subgroup (CA) showed no fibrosis with little fatty changes in its cytoplasm, while the subgroup T1A liver showed no fibrosis with uniform hepatocytes. T1A showed uniform liver architecture with no fibrosis with uniform hepatocytes. T2A showed uniform hepatocytes with no evidence of lytic necrosis or lobular inflammation. T3A showed foci of lobular inflammation, while T4A showed no fibrosis with little fatty changes inside the cytoplasm. The liver of the control subgroup (CB) showed normal architecture with focal confluent necrosis. The area of confluent necrosis with chronic inflammatory cell infiltrate replacing hepatocytes. Hepatocytes showed steatosis. T1B showed uniform hepatocytes with no evidence of lytic necrosis or lobular inflammation.

T2B showed no evidence of fibrosis or inflammation. T3B showed little foci of lobular inflammation. T4B liver showed no detected pathological changes; however, mild hydropic changes were seen in some hepatocytes (Fig. 3a- j).

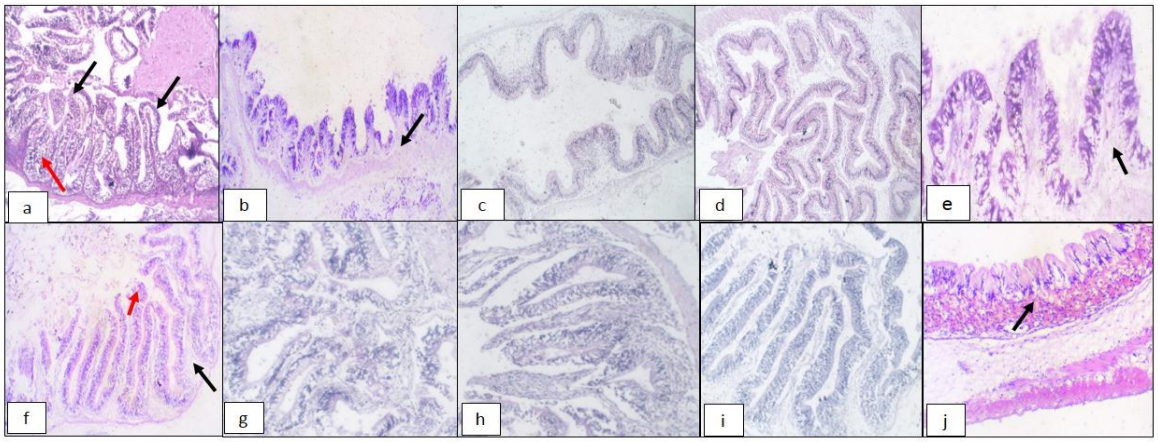

Fig. 2. (a) Control intestine (CA) showing disturbed irregular villous architecture, with no atrophy (black arrows). The lamina propria shows chronic inflammation (red arrows). Sloughed tissue is seen within the lumen (arrow heads). (H & E, 10x). **(b)** Treated subgroup (T1A) intestine displaying regular villi with no atrophy, distortion or sloughing. Lamina propria shows no edema or chronic inflammation. (H & E, 10x). **(c)** Treated subgroup (T2A) uniform intestinal villi and crypts, there is mild chronic inflammation in the lamina propria (H & E, 10x). **(d)** Normal intestinal villi from the treated subgroup (T3A) with no signs of inflammation (H & E, 10x). **(e)** Treated subgroup (T4A) regular villi with no lamina propria inflammation (black arrows). (H&E, 10x). **(f)** Control intestine (CB revealed mild disruption of intestinal villi, with chronic inflammation within the lamina propria (black arrows) (H & E, 10x). **(g)** Treated subgroup (T1B) showed irregular villi with no atrophy or inflammations (H&E, 10x). **(h)** Treated subgroup (T2B) revealed regular villi with no atrophy (H&E, 10x). **(i)** Treated subgroup (T3B) Uniform intestinal villi and crypts with mild chronic inflammation in lamina propria (H&E, 10x). **(j)** Treated subgroup (T4B) showed villi with regular and no atrophy, distortion, or sloughing. Lamina propria shows no edema or chronic inflammation. (H & E, 10x).

4.3. Spleen

Spleen of the control subgroup (CA) showed moderate proliferation of lymphoid follicles and congested sinusoids, while subgroup T1A showed mild follicular hyperplasia and sinusoids congestion. T2A showed normal white bulb with small areas of red bulb congestion. T3A showed mild hyperplasia in the white bulb with mild sinus congestion in the red bulb. T4A showed normal white and red bulbs with mild congestion. Spleen of the control subgroup (CB) showed moderate proliferation of lymphoid follicles in the white bulb and congested sinusoids. T1B showed moderate proliferation in lymphoid follicles of white bulb with increasing in red bulbs congestions. T2B showed mild lymphoid hyperplasia with little degree of red bulb congestions. T3B showed lymphoid folliciles in white pulp and sinusoids. T4B showed mild follicular hyperplasia and sinusoids congestion in the red bulb. (Fig. 4a- j).

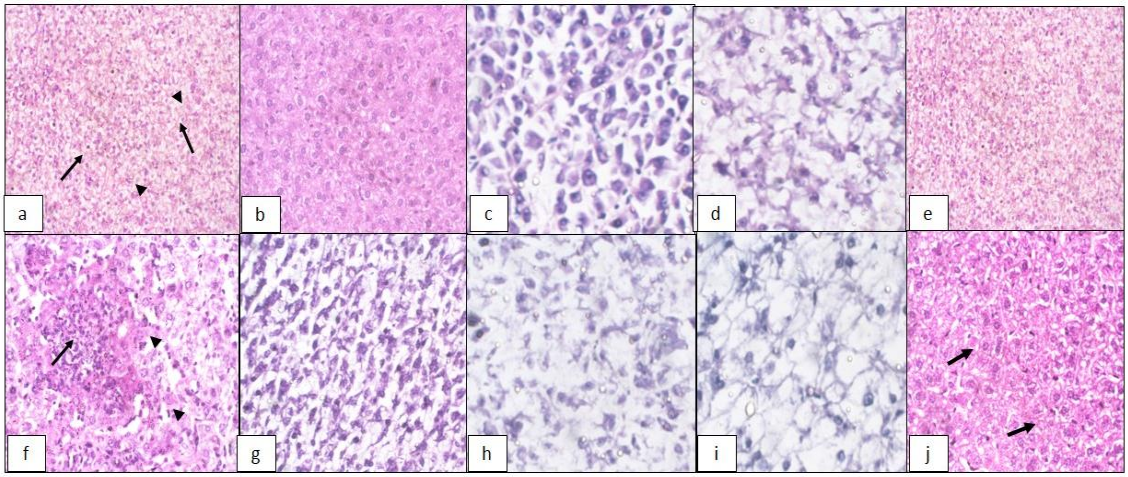

Fig. 3. (a) Uniform liver architecture of control (CA) with no fibrosis, hepatocytes with fatty changes in their cytoplasm (arrow heads) with a single focus of lytic necrosis is seen (black arrow) (H & E, 40x). **(b)** Uniform liver architecture of the treated subgroup (T1A) with no fibrosis and uniform hepatocytes (H & E, 40x). **(c)** Treated subgroup (T2A) showing uniform hepatocytes with no evidence of lytic necrosis or lobular inflammation (H & E, 40x). **(d)** (T3A) with foci of lobular inflammation (black arrows) (H & E, 40x). **(e)** Treated subgroup (T4A) revealed no fibrosis with little fatty changes inside the cytoplasm. **(f)** liver of control group (CB) showing normal architecture, with focal confluent necrosis, the area of confluent necrosis with chronic inflammatory cell infiltrate replacing hepatocytes (black arrow) (H&E, 40x). Hepatocytes show steatosis (arrow heads). **(g)** Treated subgroup (T1B) showing uniform hepatocytes with no evidence of lytic necrosis or lobular inflammation (H&E, 40x). **(h)** Treated subgroup (T2B) exhibiting no evidence of fibrosis or inflammation. **(i)** Treated subgroup (T3B) revealing little foci of lobular inflammation. **(j)** Treated subgroup (T4B) liver showig no detected pathological changes, but mild hydropic changes were seen in some of the hepatocytes (black arrows) (H & E, 40x).

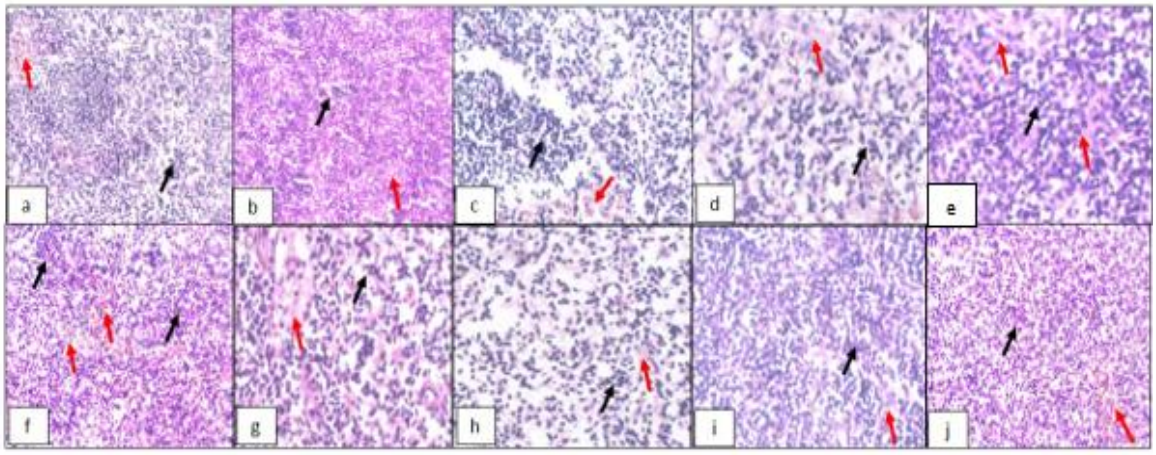

Fig. 4. (a) Spleen of control subgroup (CA) revealing moderate proliferation of lymphoid follicles, proliferated white bulb (black arrow) and congested red bulb (red arrow) (H&E, 40x). **(b)** Spleen of treated subgroup (T1A) showing mild follicular hyperplasia (black arrows) and sinusoids congestion in red bulbs (red arrow) (H&E, 40x). **(c)** Treated subgroup (T2A) displaying normal white bulb (black arrow) with small areas of red bulb congestions (red arrow) (H&E, 40x). **(d)** Treated subgroup (T3A) showing mild hyperplasia in white bulb (black arrow) with mild sinus congestion in red bulb (red arrow) (H&E, 40x), **(e)** Treated subgroup (T4A) exhibiting normal white and red bulbs with mild congestion (black and red

arrows) (H&E, 40x). **(f)** Control subgroup (CB) revealing moderate proliferation of lymphoid follicles and congested sinusoids, the proliferated white bulb (black arrows) and congested red bulb (red arrows) (H&E, 40x). **(g)** Treated subgroup (T1B) showing moderate proliferation in lymphoid follicles of white bulb (black arrow) with increasing in red bulbs congestions (red arrow) (H&E, 40x). **(h)** Treated subgroup (T2B) revealing mild lymphoid hyperplasia with little degree of red bulb congestions (black and red arrows) (H&E, 40x). **(i)** Treated subgroup (T3B) showing lymphoid follicles in white pulp (black arrows) and sinusoids (red arrows) (H&E, 40x), **(j)** Treated subgroup (T4B) showing mild follicular hyperplasia and sinusoids congestion, the follicular hyperplasia (black arrows) and sinusoids congestion (red bulb) (H&E, 40x).

5. Oxidative biomarkers and antioxidant enzymes before and after experimental infection with A. hydrophila in both environments (A) and (B)

5.1. Before A. hydrophila infection

Group (A) in Table (5) shows that, all oxidative enzymes recorded a highly significant difference between subgroups ($P \leq 0.05$). MDA recorded a high significance in T1 and control subgroups. SOD and GSH recorded highly significant values in subgroups T3. GPx activity recorded highly significant difference in T4 subgroup. CAT enzyme activity showed a highly significant difference among subgroups, with no clear variations between them.

For group (B) in Table (5), all oxidative enzymes recorded a highly significant difference between subgroups ($P \leq 0.05$). MDA showed higher values in T1 and T2 subgroups. SOD, GSH and GPx oxidative parameters activities showed highly significant difference in T3 subgroup. CAT enzyme activity showed a highly significant difference in T4 and T1 values.

Table 5. Oxidative biomarkers and antioxidant enzymes in *O. niloticus* feeding *P. pavonica* in environments (A) and (B)

Subgroups / Factors		*Padina pavonica* (g/kg diet)					*P. Value*
		(0) Control	2.0 (T1)	4.0 (T2)	6.0 (T3)	8.0 (T4)	
MDA nmol/g.tissue	A	61.40±0.34 ^{b}	128±0.45^{a}	51.90±0.46 ^{c}	28.10±0.46 ^{e}	41±1.05 ^{d}	0.00
	B	147.70±1.89 ^{b}	153.50±0.69 ^{a}	153.20±0.87 ^{a}	150.90±0.59 ^{ab}	80.20±1.51 ^{c}	0.00
SOD U/g. tissue	A	1245.50±1.45 ^{c}	809.30±1.09 ^{d}	1312.10±1.22 ^{b}	2369.50±2.11 ^{a}	1244.50±1.68 ^{c}	0.00
	B	1875.60±1.75 ^{c}	1937.40±2.16 ^{b}	1938±2.35 ^{b}	1994.50±0.85 ^{a}	1807±1.29 ^{d}	0.00
GSH mg. /g. tissue	A	0.98±0.04 ^{e}	1.50±0.06 ^{d}	2.99±0.04 ^{b}	3.78±0.04 ^{a}	2±0.04 ^{c}	0.00
	B	7.40±0.04 ^{d}	12.90±0.35 ^{b}	8.39±0.09 ^{c}	19.10±0.43 ^{a}	12.19±0.16 ^{b}	0.00
GPx U/g. tissue	A	350±0.87 ^{c}	329.80±1.07 ^{d}	369.30±1.12 ^{b}	350.20±1.39 ^{c}	388.30±1.52^{a}	0.00
	B	77±0.76 ^{d}	96.80±0.59 ^{c}	57.90±0.89 ^{e}	329.50±1.08 ^{a}	290.10±1.24 ^{b}	0.00
CAT U/g. tissue	A	0.57±0.04 ^{b}	0.50±0.04 ^{b}	1±0.04 ^{a}	0.96±0.05 ^{a}	1.04±0.04 ^{a}	0.00
	B	0.50±0.04 ^{b}	1±0.04 ^{a}	0.50±0.04 ^{b}	0.50±0.04 ^{b}	1.02±0.04 ^{a}	0.00

Note that: a-d means there was statistically significant difference between values at $P \leq 0.05$ in the same row. (n=10), one way ANOVA test was used to compare means.

5.2. After A. hydrophila infection

Concerning group (A) in Table (6), all oxidative enzymes recorded a highly significant difference between subgroups ($P \leq 0.05$). MDA showed highly significant difference in T3 and T4 subgroups. SOD and GSH showed a highly significant difference in T3 subgroup. GPx and CAT activities showed highly significant difference in T4 subgroup.

Concerning group (B) in Table (6), all oxidative enzymes recorded a highly significant difference between subgroups ($P \leq 0.05$). MDA showed a highly significant difference in T4 subgroup. SOD and GSH showed highly significant differences in T4 subgroup. GPx and CAT activities showed that a highly significant difference in subgroups of T2, T3 and T4.

Table 6. Oxidative biomarkers and antioxidant enzymes in *O. niloticus* after challenging with *A. hydrophila* in environments (A) and (B)

Subgroups / Factor		*Padina pavonica* (g/kg diet)					*P. Value*
		(0) Control	2.0 (T1)	4.0 (T2)	6.0 (T3)	8.0 (T4)	
MDA nmol/g.tissue	A	228.30±1.34^{e}	306.10±1.8^{d}	362.10±1.52^{c}	370.90±1.17^{a}	366.50±1.14^{b}	0.00
	B	143.10±0.91^{e}	365.40±0.97^{b}	315.60±0.73^{c}	294.60±1.24^{d}	368.20±0.76^{a}	0.00
SOD U/g.tissue	A	1312±1.17^{d}	1117.90±1.75^{e}	4680.70±1.69^{c}	7864.40±2.01^{a}	7117.70±1.56^{b}	0.00
	B	3743.50±1.63^{c}	2680±1.76^{e}	4305.60±2.11^{b}	2991.70±1.08^{d}	7115.80±1.05^{a}	0.00
GSH mg/g.tissue	A	12.49±0.12^{d}	5.42±0.08^{e}	27.70±0.97^{b}	37.50±1.01^{a}	23.43±0.11^{c}	0.00
	B	5.46±0.09^{e}	15.13±0.09^{b}	9.89±0.10^{c}	7.25±0.09^{d}	22.10±0.75^{a}	0.00
GPx U/g. tissue	A	232.80±1.36^{c}	19.70±0.54^{e}	174.30±1.32^{d}	275.10±1.08^{b}	311.50±0.91^{a}	0.00
	B	39.30±1.04^{d}	58±0.84^{c}	175.60±1.08^{a}	77.70±0.72^{b}	77.80±0.94^{b}	0.00
CAT U/g. tissue	A	0.07±0.00^{c}	0.14±0.01^{bc}	0.21±0.07^{b}	0.50±0.04^{a}	0.49±0.03^{a}	0.00
	B	0.05±0.01^{c}	0.46±0.01^{a}	0.21±0.03^{b}	0.28±0.04^{b}	0.49±0.03^{a}	0.00

Note that: a-e means there was statistically significant difference between values at $P \leq 0.05$ within the same row. (n=10); one way ANOVA test to compare means

6. Lysozyme activity results in *O. niloticus* before and after challenge with A. hydrophila in environments (A) and (B)

6.1. Before challenging with A. hydrophila

From Table (7), groups A and B show that lysozyme enzyme activities recorded highly significant difference within subgroups ($P \leq 0.05$), while the higher values were recorded in T4 subgroup, followed by T3.

6.2. After challenging with A. hydrophila

Table (7) shows groups A and B with lysozyme enzyme activities recording a highly significant difference among subgroups ($P \leq 0.05$), while higher values were recorded in control subgroup, followed by T1.

Table 7. Lysozyme activity results in *O. niloticus* before and after challenge with *A. hydrophila* in environments (A) and (B)

Subgroups / Factor		*Padina pavonica* (g/kg diet)					*P*-value
		(0) Control	2.0 (T1)	4.0 (T2)	6.0 (T3)	8.0 (T4)	
Lysozyme activity before challenge	A	118.50±4.03^{e}	191.9±1.85^{c}	164.23±3.03^{d}	211.79±2.51^{b}	276.75±2.49^{a}	0.00
	B	164.96±1.93^{d}	167.67±2.59^{d}	197.81±5.91^{c}	209.33±2.8^{b}	256.07±2.36^{a}	0.00
Lysozyme activity after challenge	A	263.08±1.17^{a}	220.15±1.24^{b}	195.02±1.36^{c}	196.12±1.18^{c}	167.41±2.23^{d}	0.00
	B	237.57±2.04^{a}	216.65±1.28^{b}	138.84±1.18^{e}	205.66±1.34^{c}	190.35±2.53^{d}	0.00

Note that: a-e means there was statistically significant difference between values at $P \leq 0.05$ within the same row. (n=10), one way ANOVA test to compare means.

7. Mortality after challenging with *A. hydrophila* in environments (A) and (B)

The total number of fish starting the challenge trial was 10 for each subgroup in both environments A and B. A low mortality rate was detected between subgroups. Some subgroups recorded zero mortality rate as T2A and T1B, while others recorded only one dead fish individual (10%) after the end of the experimental period as CA, T1A, CB and T4B subgroups. Some subgroups recorded 2 dead fish (20%) as T3A, T4A and T3B. The higher mortalities were at T2B subgroup, with 3 dead fish (30%).

DISCUSSION

Recently, most researchers have investigated various aspects of the administration of effective feed additives to fish, which are affecting growth performance and health status through adjusting the microbial balance in the intestine **(Van Doan *et al.*, 2020)**. In this context, the current study was introduced to perform the beneficial effects of *Padina pavonica* brown alga as a feed additive on the fish body composition and oxidative enzymes as a biomarker for the immune enhancement of fish.

Upon analyzing the alga structure it was found that the presence of alginates and fucoidan/ alginic acid residues are similar to the data presented in the study of **Fernando *et al.* (2017)**. Moreover, mannuronic and guluronic acids were regarded as a basic polysaccharide backbone in the FT-IR analysis, which matches the results of **Pereira *et al.* (2013)**. Additionaaly, the occurrence of sulfate groups and galactose rings in algal polysaccharides is attributed to their role in biological activities and their varying sugar levels **(Hong *et al.*, 2021)**.

Fish body composition represents the measurements of water, protein, fat and ash composition as a good evidence of fish health, quality and physiological conditions **(Love, 1997)**. A significant increase was recorded in the values of moisture, protein and ash aligned with a decrease in lipid concentrations in experimental group (A), which was fed a higher *P. pavonica* concentration (T4). In group (B), only T4 recorded significant

increases in moisture and protein values; these results agree with those of **Younis *et al.* (2018)**, who recorded similar increases in moisture, protein and ash while the reverse was true in the lipid concentrations of *O. niloticus* fed higher algal concentrations (20% and 40%), compared to the control group which was fed the red algae *Gracilaria arcuata* meal. **Soler-Vila *et al.* (2009)** detected an increase in protein levels of rainbow trout (*O. mykiss*) in the group fed a diet containing a higher concentration of the red alga, *P. dioica* (10%). The decreasing lipid levels in both groups (A and B) fed on a higher *P. pavonica* meal coincides with the finding of **Valente *et al.* (2006)** who recorded that, the lipid value of European seabass, (*Dicentrarchus labrax*) juveniles fed a *Gracilaria cornea* diet decreased with 10% of gracilaria, compared to both control and 5% diet.

The present results showed highly significant increases in GSI values in both fish groups of A and B, where the higher values were recorded with higher feeding concentration (T4) that means the effect of using brown alga *P. pavonica* as immunostimulant may be directed to another pathway as sexual effect and increasing the efficiency of gonads. Similar results are those of **Thépot *et al.* (2021)**, who postulated an increase in GSI in the rabbitfish fed on the red seaweed *Asparagopsis taxiformis* in different forms (whole, methanolic extract and residue of extraction) and dietary inclusion levels of 1.5, 3 and 6% of feed. On the other hand, in T4 of experimental group (A), a significant increase in HSI values was observed; however, no significant difference was recorded among the farming environment group (B). These findings may be due to hypertrophy or hyperplasia of liver cells **(Ayoola, 2008)**. The present data nearly concur with those of **Mahmoud *et al.* (2018)**, who detected an increase in the HSI of *O. niloticus* upon feeding with spirulina (2%). For the SSI, no significant differences ($P > 0.05$) were recorded in experimental group (A), while the farming condition group (B) registered a highly significant difference ($P \leq 0.05$) in subgroup T4. This , in turn, indicates the positive effect of *P. pavonica* on splenic tissue as an immune organ. Partly similar results are those reported in the study of **Abd El-daim *et al.* (2021)**, who regarded an increase in the SSI values in *O. niloticus* fed a mixture of immunostimulants *Azolla nilotica* (AZN) of 5% and *Spirulina platensis* (SP) of 1% group.

In addition, an increase was recorded in non-enzymatic marker of oxidative status MDA, revealing a possible oxidative damage to the cells after pathogenic bacterial infections. In this study, MDA activities were significantly decreased in all subgroups fed highly doses (T3 and T4) of *P. pavonica* alga in both environments of the experiment before and after bacterial infection with *A. hydrophila*, which indicates that the alga increased the immune responses and decreased the damaging effect of MDA. The cells'damage may be attributed to the presence of pathogenic bacteria *A. hydrophila*, which subsequently increased MDA levels. In this context, **Baldissera *et al.* (2019)** stated that, the infection of silver catfish (*Rhamdia quelen*) by *A. caviae* increased the hepatic and renal MDA levels after four days of infection.

As shown in the results, the oxidative biomarkers and antioxidant enzymes SOD, GSH, GPx and CAT for both environments of A and B recorded a significant increase in the subgroups fed high algal doses (T3 and T4). This finding agree with those of **Rajendran *et al.* (2016)** who proved that fish fed with macroalga, *Padina gymnospora,* stimulated the immune response of common carp (*Cyprinus carpio*). After *A. hydrophila* infections for all subgroups, results showed higher oxidative enzyme activities in the subgroups fed higher algal doses (T3 and T4), which reveals no harmful effect of pathogenic bacteria on the cells immunized with natural immune enhancer as algae. This outcome coincides with that of **Pal *et al.* (2019)** about SOD and CAT enzymes which increased after the infection of rohu (*Labeo rohita*) with *A. hydrophila*. Notably, SODs can prevent oxidative stress by catalyzing the dismutation reaction of O_2 to oxygen and hydrogen peroxide (H2O2) in the living organisms **(Bandeira Junior & Baldisserotto, 2021)**. The activity of CAT enzyme is usually consumed by cells in every organ; by activating the liver to rapidly detoxify the harmful H_2O_2 into less reactive O_2 and H_2O molecules, CAT enzyme values increased inside the cells, and hence the immune efficiency increased. However, the results disagree with what stated about *Oreochromis niloticus* infections with *A. hydrophila* which caused decreases in SOD and CAT activities **(Moustafa *et al.*, 2020)**. On the other hand, some researchers reported no effective activity for SOD or CAT in the infected fish cells with pathogenic *A. hydrophila* as in gibel carp (*Carassius auratus gibelio*) (**Cao *et al.*, 2018; Zhang *et al.*, 2018; Zhang *et al.*, 2019)**.

The present results recorded an increase in GSH and GPx in both environments (A and B), with significantly high values in subgroups fed with highly algal doses (T3 and T4). The same results appeared after *A. hydrophila* infection, indicating the powelful effect of *P.pavonica* alga on the oxidative biomarkers that were not affected by the pathogenic bacteria. According to the results, GSH has the ability to attack cellular ROS (reactive oxygen species) and produce a co-factor for GPx in a reduction reaction **(Backos *et al.*, 2012)**. GSH levels did not show either an increase or a decrease during the previous studies; a finding which matches with that of **Tang *et al.* (2019)** detected decreasing levels of the intestinal GSH in 3 and 12h post-infection; however, an increase was recorded in the intestinal GSH levels in 6 and 48h post-infection in common carp (*Cyprinus carpio*) after challenging with *A.hydrophila*. In this respect, **Vinosha *et al.* (2020)** reported a decrease in the hepatic GSH levels in Nile tilapia after infection. It is worthy to mention that, the GSH values could vary due to different factors, such as fish species, bacterial species and the immunenhancer substances included in the fish meal.

As observed in the results, lysozyme enzyme levels in both experimental groups of A and B showed a significant increase in subgroups of T3 and T4, compared to the control subgroups. This points out the immunestimulant effect of *P.pavonica* brown algae on such subgroups. **Swain *et al.* (2006)** reported that, the immunostimulants are naturally occurring compounds that acts on the non-specific defence mechanisms or the specific

immune reactions of the host to handle pathogens. Lysozyme activity usually changes according to the type of immunostimulants to which fish are exposed, and thus the increase in lysozyme following immunostimalation has been detected in different fish species, and this finding is related to increase in the protection against pathogens **(Saurabh & Sahoo, 2008)**. On the other hand, lysozyme enzyme activity after being challenged with *A. hydrophila* bacteria was significantly reduced in T4 subgroup of A and B environments when compared to control subgroup. This may indicate the exhusion of lysozyme in facing *A.hydrophila* infection. This opinion agrees with that of **Lie *et al.* (1989)** who explained that, the reduction in lysozyme levels measured in Atlantic salmon diseased with 'Hitra-disease' or cold-water vibriosis results from increasing susceptibility to the disease as postmortem degradation of the lysozyme levels. The present results suggested that, the maximum lysozyme activity recorded in both environments of A and B before challenge test in subgroup T4 seems to be optimum in immunostimulation and reduction of mortality, which decreases for the same subgroup after bacterial infection, subsided with an increasing mortality rate among subgroups.

Histopathological changes between the control and treated subgroups in both environments showed no significant changes between them due to the moderate to mild effect of the brown algae (*Padina pavonica*) on the internal organs (intestine, liver and spleen). This result agrees with that of **Hussein (2017)**, who found no histopathological changes between control and treated groups in the liver and/or intestine samples of *O. niloticus* fed seaweed *Taonia atomaria* supplementation.

From the previous studies, the most effective bacterial species was *A. hydrophila*, because it is one of the most prevalent species of fish populations causing septicemic disorders **(Sandeep, 2016)**. The decreased mortality rate in both environment after feeding with *P.pavonica* alga can be due to the increase in the immune responses against the pathogenic bacteria. This finding concurs wih that of **Das *et al.* (2015)** who noticed an increasing disease resistance against *A. hydrophila* after feeding on the natural leaf extract of *Ocimum sanctum* in two different fishes *Oreochromis mossambicus* and *Labeo rohita*. Furthermore, *Padina gymnospora* significantly decreased the mortality rate of *C. carpio* after being challenged with *A. hydrophila* and *E. tarda* **(Rajendran *et al.*, 2016)**.

CONCLUSION

Given the recorded results, it can be deduced that, using brown alga *P. pavonica* as a fish meal in aquacultures at the recommended dose (8.0 g/kg food) increases fish productivity while improving immunity and defence against pathogenic bacteria *A. hydrophila*.

REFERENCES

Abd El-daim, A.; El Asely, A.; Kandiel, M.; Abd El-Gawad, E.; Elabd, H.; Shaheen, A., and Abbass, A. A. A. (2021). Effect of Spirulina platensis and Azolla nilotica as feed additives on growth performance, antioxidant enzymes and fecundity of Oreochromis niloticus. *Iran. J. Fish. Sci., 20*(3): 846-862. doi: 10.22092/ijfs. 2021.124136

Ansari, A. A.; Ghanem, S. M. and Naeem, M. (2019). Brown Alga Padina: A review. *Int. j. botany stud., 4*(1): 01-03.

Ayoola, S. (2008). Histopathological Effect of Glyphosate on Juvenile African Catfish (Clarias gariepinus). *American-Eurasian J. Agric. & Environ. Sci, 4*(3): 362-367.

Backos, D. S.; Franklin, C. C. and Reigan, P. (2012). The role of glutathione in brain tumor drug resistance. *Biochem. Pharmacol., 83*(8): 1005-1012. doi: https://doi.org/10.1016/j.bcp.2011.11.016

Baldissera, M. D.; Souza, C. F.; Abbad, L. B.; Verdi, C. M.; Santos, R. C. V.; da Silva, A. S. and Baldisserotto, B. (2019). Dietary supplementation with caffeine increases survival rate, reduces microbial load and protects the liver against Aeromonas hydrophila-induced hepatic damage in the grass carp Ctenopharyngodon idella. *Microb. Pathog., 135*: 103-637. doi: https://doi.org/10.1016/j.micpath.2019.103637

Bancroft, J.; Stevens, A. and Turner, D. (1996). *Theory and practice of histological techniques 4th Ed Churchill Living Stone, New York Edinburgh*.

Bandeira Junior, G. and Baldisserotto, B. (2021). Fish infections associated with the genus Aeromonas: a review of the effects on oxidative status. *J. Appl. Microbiol., 131*(3): 1083-1101. doi: https://doi.org/10.1111/jam.14986

Beers, R. F. and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem., 195*(1): 133-140.

Beutler, E. (1971). Reduced glutathione (GSH). *Red Cell Metab:* 103-105. doi: https://ci.nii.ac.jp/naid/10024866629/en/

Cao, S.; Zhang, P.; Zou, T.; Fei, S.; Han, D.; Jin, J.; Liu, H.; Yang, Y.; Zhu, X. and Xie, S. (2018). Replacement of fishmeal by spirulina Arthrospira platensis affects growth, immune related-gene expression in gibel carp (Carassius auratus gibelio var. CAS III), and its challenge against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol. 79*: 265-273. doi: https://doi.org/10.1016/j.fsi.2018.05.022

Das, R.; Raman, R. P.; Saha, H. and Singh, R. (2015). Effect of Ocimum sanctum Linn. (Tulsi) extract on the immunity and survival of Labeo rohita (Hamilton) infected with *Aeromonas hydrophila*. *Aquac. Res., 46*(5): 1111-1121. doi: https://doi.org/10.1111/are.12264

Dawood, M. A.; El Basuini, M. F.; Zaineldin, A. I.; Yilmaz, S., Hasan; M., Ahmadifar, E.; El Asely, A. M.; Abdel-Latif, H. M.; Alagawany, M. and Abu-Elala, N. M. (2021). Antiparasitic and antibacterial functionality of essential oils: an alternative approach for sustainable aquaculture. *Pathogens, 10*(2): 185. doi: https://doi.org/10.3390/pathogens10020185

Del Rio, D.; Pellegrini, N.; Colombi, B.; Bianchi, M.; Serafini, M.; Torta, F.; Tegoni, M.; Musci, M. and Brighenti, F. (2003). Rapid Fluorimetric Method to Detect Total

Plasma Malondialdehyde with Mild Derivatization Conditions. *Clin. Chem., 49*(4): 690-692. doi: https://doi.org/10.1373/49.4.690

Drury, R.and Wallington, E. (1987). *Histological Techniques. Oxford University Press*: Oxford, New York, Toronto.

Dytham, C. (2011). *Choosing and using statistics: a biologist's guide*: John Wiley & Sons.

Ellis, A. (1990). Lysozyme assays–In: Techniques in Fish Immunology, vol. 1 (Eds) JS Stolen, TC Fletcher, DP Anderson, BS Robertson, WB Van Muiswinkel (pp. 101-103): SOS Publications, Fair Haven, USA.

Fernando, I.; Sanjeewa, K. K. A.; Samarakoon, W.; Lee, W. W.; Kim, H.-S.; Kim, E.-A., Gunasekara, U., Abeytunga, D., Nanayakkara, C., and De Silva, E. (2017). FTIR characterization and antioxidant activity of water soluble crude polysaccharides of Sri Lankan marine algae. *Algae, 32*(1): 75-86. doi: https://doi.org/10.4490/algae.2017.32.12.1

Folch, J.; Lees, M.; and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem., 226*(1): 497-509.

Gamal-Eldeen, A. M.; Ahmed, E. F.; and Abo-Zeid, M. A. (2009). In vitro cancer chemopreventive properties of polysaccharide extract from the brown alga, Sargassum latifolium. *Food Chem. Toxicol., 47*(6): 1378-1384. doi: https://doi.org/10.1016/j.fct.2009.03.016

Hong, T.; Yin, J.-Y.; Nie, S.-P.; and Xie, M.-Y. (2021). Applications of infrared spectroscopy in polysaccharide structural analysis: Progress, challenge and perspective. *Food Chemistry: X, 12*: 100168. doi: https://doi.org/10.1016/j.fochx.2021.100168

Hussein, E. E.-S. (2017). Effect of seaweed supplemented diets on Nile tilapia, Oreochromis niloticus performance. *Int. j. fish. aquat, 5*: 205-210.

Kosanić, M.; Ranković, B.; and Stanojković, T. (2015). Biological activities of two macroalgae from Adriatic coast of Montenegro. *Saudi J. Biol. Sci., 22*(4): 390-397. doi: https://doi.org/10.1016/j.sjbs.2014.11.004

Lie, Ø.; Evensen, Ø.; Sorensen, A.; and Froysadal, E. (1989). Study on lysozyme activity in some fish species. *Dis. Aquat. Org., 6*(1): 1-5. doi: 10.3354/dao006001

Love, R. (1997). Biochemical dynamics and the quality of fresh and frozen fish *Fish processing technology* (pp. 1-31): Springer.

Maghawri, A.; Marzouk, S. S.; Ezz El-Din, H. M.; and Nashaat, M. (2023). Effect of brown algae Padina pavonica as a dietary supplement on growth performance and health status of cultured Oreochromis niloticus. *Egypt. J. Aquat. Res.*. doi: https://doi.org/10.1016/j.ejar.2023.03.001

Mahmoud, M. M. A.; El-Lamie, M. M. M.; Kilany, O. E.; and Dessouki, A. A. (2018). Spirulina (Arthrospira platensis) supplementation improves growth performance, feed utilization, immune response, and relieves oxidative stress in Nile tilapia (Oreochromis niloticus) challenged with *Pseudomonas fluorescens*. *Fish Shellfish Immunol., 72*: 291-300. doi: https://doi.org/10.1016/j.fsi.2017.11.006

Moustafa, E. M.; Dawood, M. A. O.; Assar, D. H.; Omar, A. A.; Elbialy, Z. I.; Farrag, F. A.; Shukry, M.; and Zayed, M. M. (2020). Modulatory effects of fenugreek seeds powder on the histopathology, oxidative status, and immune related gene

expression in Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila*. *Aquaculture, 515*, 734589. doi: https://doi.org/10.1016/j.aquaculture.2019.734589

Ohkawa, H.; Ohishi, N.; and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem., 95*(2): 351-358. doi: https://doi.org/10.1016/0003-2697(79)90738-3

Pal, S.; Roy, D.; Ray, S. D.; and Homechaudhuri, S. (2019). *Aeromonas hydrophila* induced mitochondrial dysfunction and apoptosis in liver and spleen of Labeo rohita mediated by calcium and reactive oxygen species. *Turkish J. Fish. Aquat. Sci., 20*(4): 255-266. doi: http://doi.org/10.4194/1303-2712-v20_4_02

Pandit, D. N.and Gupta, M. (2019). Heapto-somatic index, gonado-somatic index and condition factor of Anabas testudineus as bio-monitoring tools of nickel and chromium toxicity. *Int. j. innov. eng. technol. 12*(3): 25-28. doi: http://dx.doi.org/10.21172/ijiet.123.05

Paoletti, F.; Aldinucci, D.; Mocali, A.; and Caparrini, A. (1986). A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal. Biochem., 154*(2): 536-541. doi: https://doi.org/10.1016/0003-2697(86)90026-6

Pereira, L.; Gheda, S. F.; and Ribeiro-Claro, P. J. (2013). Analysis by vibrational spectroscopy of seaweed polysaccharides with potential use in food, pharmaceutical, and cosmetic industries. *Int. J. Carbohydr. Chem., 2013*. doi: http://dx.doi.org/10.1155/2013/537202

Rajendran, P.; Subramani, P. A.; and Michael, D. (2016). Polysaccharides from marine macroalga, Padina gymnospora improve the nonspecific and specific immune responses of *Cyprinus carpio* and protect it from different pathogens. *Fish. Shellfish. Immunol., 58*: 220-228. doi: https://doi.org/10.1016/j.fsi.2016.09.016

Ran, C.; Qin, C.; Xie, M.; Zhang, J.; Li, J.; Xie, Y.; Wang, Y.; Li, S.; Liu, L.; Fu, X.; Lin, Q.; Li, N.; Liles, M. R.; and Zhou, Z. (2018). *Aeromonas veronii* and aerolysin are important for the pathogenesis of motile aeromonad septicemia in cyprinid fish. *Environ. Microbiol., 20*(9): 3442-3456. doi: https://doi.org/10.1111/1462-2920.14390

Sahoo, D.; Tang, X.; and Yarish, C. (2002). Porphyra – the economic seaweed as a new experimental system. *Curr. Sci., 83*(11): 1313-1316. doi: https://www.jstor.org/stable/24106954

Sandeep, G. (2016). Pan-genome analysis of *Aeromonas hydrophila*, *Aeromonas veronii* and *Aeromonas caviae* indicates phylogenomic diversity and greater pathogenic potential for *Aeromonas hydrophila*. *Antonie van Leeuwenhoek, v. 109*(no. 7): pp. 945-956-2016 v.2109 no.2017. doi: http://dx.doi.org/10.1007/s10482-016-0693-6

Saputra, F.; Shiu, Y.-L.; Chen, Y.-C.; Puspitasari, A. W.; Danata, R. H.; Liu, C.-H.; and Hu, S.-Y. (2016). Dietary supplementation with xylanase-expressing B. amyloliquefaciens R8 improves growth performance and enhances immunity against Aeromonas hydrophila in Nile tilapia (*Oreochromis niloticus*). *Fish. Shellfish. Immunol., 58*: 397-405. doi: https://doi.org/10.1016/j.fsi.2016.09.046

Saurabh, S.and Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. *Aquac. Res., 39*(3): 223-239. doi: https://doi.org/10.1111/j.1365-2109.2007.01883.x

Soler-Vila, A.; Coughlan, S.; Guiry, M. D.; and Kraan, S. (2009). The red alga *Porphyra dioica* as a fish-feed ingredient for rainbow trout (*Oncorhynchus mykiss*): effects on growth, feed efficiency, and carcass composition. *J. Appl. Phycol., 21*(5): 617-624. doi: 10.1007/s10811-009-9423-z

Subramanian, G.; Nagaraj, A.; Gunavathi, P.; Jamuna, S.; Banumathi, V.; Jayanthi, V.; Manivannan, M.; Kanaga, V.; and Ravi, P. (2015). Biochemical Composition of *Padina pavonica* (L.) a Brown Alga from Mandapam Coastal Regions; Southeast Coast of India. *IJAIDR, 2*: 21-24.

Swain, P.; Sahoo, P. K.; and Ayyappan, S. (2006). *Fish. shellfish. Immunol.: An introduction*: Narendra Publishing House.

Tang, Y.; Han, L.; Chen, X.; Xie, M.; Kong, W.; and Wu, Z. (2019). Dietary Supplementation of Probiotic *Bacillus subtilis* Affects Antioxidant Defenses and Immune Response in Grass Carp Under *Aeromonas hydrophila* Challenge. *Probiotics.antimicrob.proteins, 11*(2): 545-558. doi: https://doi.org/10.1007/s12602-018-9409-8

Thépot, V.; Campbell, A. H.; Rimmer, M. A.; and Paul, N. A. (2021). Effects of a seaweed feed inclusion on different life stages of the mottled rabbitfish *Siganus fuscescens. Aquac. Res., 52*(12): 6626-6640. doi: https://doi.org/10.1111/are.15533

Valente, L. M. P.; Gouveia, A.; Rema, P.; Matos, J.; Gomes, E. F.; and Pinto, I. S. (2006). Evaluation of three seaweeds *Gracilaria bursa-pastoris, Ulva rigida* and *Gracilaria cornea* as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture, 252*(1): 85-91. doi: https://doi.org/10.1016/j.aquaculture.2005.11.052

Van Doan, H.; Hoseinifar, S. H.; Ringø, E.; Ángeles Esteban, M.; Dadar, M.; Dawood, M. A. O.; and Faggio, C. (2020). Host-Associated Probiotics: A Key Factor in Sustainable Aquaculture. *Reviews in Fisheries Science & Aquaculture, 28*(1): 16-42. doi: https://doi.org/10.1080/23308249.2019.1643288

Vinosha, M.; Palanisamy, S.; Anjali, R.; Li, C.; Yelithao, K.; Marudhupandi, T.; Tabarsa, M.; You, S.; and Prabhu, N. M. (2020). Sulfated galactan from *Halymenia dilatata* enhance the antioxidant properties and prevents *Aeromonas hydrophila* infection in tilapia fish: In vitro and in vivo study. *Int. J. Biol. Macromol., 158*: 569-579. doi: https://doi.org/10.1016/j.ijbiomac.2020.04.212

Wang, K.; Fang, X.; Ma, N.; Lin, Q.; Huang, Z.; Liu, W.; Xu, M.; Chen, X.; Zhang, W.; and Zhang, Y. (2015). Myeloperoxidase-deficient zebrafish show an augmented inflammatory response to challenge with *Candida albicans*. *Fish. Shellfish. Immunol., 44*(1): 109-116. doi: https://doi.org/10.1016/j.fsi.2015.01.038

Younis, E.-S. M.; Al-Quffail, A. S.; Al-Asgah, N. A.; Abdel-Warith, A.-W. A.; and Al-Hafedh, Y. S. (2018). Effect of dietary fish meal replacement by red algae, *Gracilaria arcuata*, on growth performance and body composition of Nile tilapia *Oreochromis niloticus*. *Saudi J. Biol. Sci., 25*(2): 198-203. doi: https://doi.org/10.1016/j.sjbs.2017.06.012

Zaim, S.; Mortadi, A.; Chibi, F.; Benchennouf, E. H.; Arsalane, W.; Cherkaoui, O., Rchid, H.; Nmila, R.; and El Moznine, R. (2020). Extraction of polysaccharides from brown algae: rheological studies. *Iran. Polym. J., 29*(12): 1137-1145. doi: https://doi.org/10.1007/s13726-020-00867-9

Zhang, P.; Cao, S.; Zou, T.; Han, D.; Liu, H.; Jin, J.; Yang, Y.; Zhu, X.; Xie, S.; and Zhou, W. (2018). Effects of dietary yeast culture on growth performance, immune response and disease resistance of gibel carp (*Carassius auratus gibelio* CAS Ⅲ). *Fish. Shellfish. Immunol., 82*: 400-407. doi: https://doi.org/10.1016/j.fsi. 2018. 08.044

Zhang, P.; Fu, L.; Liu, H.; Huda, N.-U.; Zhu, X.; Han, D.; Jin, J.; Yang, Y.; Kim, Y.-S.; and Xie, S. (2019). Effects of inosine 5′-monophosphate supplementation in high fishmeal and high soybean diets on growth, immune-related gene expression in gibel carp (*Carassius auratus gibelio* var. CAS Ⅲ), and its challenge against *Aeromonas hydrophila* infection. *Fish. Shellfish. Immunol., 86*: 913-921. doi: https://doi.org/10.1016/j.fsi.2018.12.016