ISSN- 2394-5125 Vol 8, Issue 1, 2021

GROWTH PERFORMANCE, ANTIOXIDANT ENZYME ACTIVITIES AND METABOLIC RESPONSE OF NILE TILAPIA (*Oreochromis niloticus)* FED DIET WITH DIFFERENT LEVELS OF RAW AND FERMENTED AZOLLA (*Azolla filiculoides)* FOLLOWING THERMAL STRESS

LAILA M. GALLEGO[1*], YEW-HU CHIEN[1], ISAGANI P. ANGELES JR.[2]

[1]College of Life Sciences, Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan 202, Republic of China
[2]Institute of Fisheries/Freshwater Fisheries Center, Isabela State University, San Fabian, Echague, Isabela, Philippines 3309

***Corresponding Author**: lailagallego1010@gmail.com

ABSTRACT: This study evaluated dietary effects on growth performance, antioxidant capacity and metabolic response of Nile tilapia *Oreochromis niloticus* fed diet with raw azolla (RA) and fermented azolla (FA) following thermal stress. Fish were fed diet with different levels of RA [10% (10RA); 20% (20RA); 30% (30RA); 40% (40RA)], FA [10% (10FA); 20% (20FA); 30% (30FA); 40% (40FA)] and a control diet (C) without azolla for ten weeks. Growth and survival were monitored periodically. After rearing, fish were subjected to different temperature (low: 25°C, medium: 28°C and high: 33°C), and after a week, antioxidant capacity [superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase(GR)] and metabolic response [glucose (Gluc), triglycerides (Trig) and lactate (Lac)] were analyzed. The growth performance of fish fed diet with 20RA, 20FD and 30FD were better as compared to C. In addition, fish fed with 10-40% FA had a better FCR as compared to C. Disregarding levels, fish fed with FA showed better %Survival after the feeding trial (74%). No significant difference was found on antioxidant capacity (SOD, GPx, GR) and metabolic response (Gluc, trig, Lac) of fish among treatments. The activities of antioxidant capacity and metabolic response of Nile tilapia against thermal stress showed no interaction effect between levels and temperature. Overall, inclusion of 20RA and 20-30FD in the diet could be a potential feed ingredient in the diet of Nile tilapia.

KEYWORDS: Azolla, Antioxidants, Growth performance, Metabolic response, Nile tilapia, Thermal stress

I. INTRODUCTION

Search for natural antioxidants as alternatives to synthetic products is of great interest, particularly in the aquaculture industry. Natural aquatic plants have been reported to have significant effects on growth, feed intake and reproduction of fish [17]. The cost of purchasing fish feed is quite high, so an alternative ingredient is needed that is cheap, easily available and environmental friendly [44]. The right feed ingredients can be extracted from water weeds, but to date, water weeds have been considered as waste; thus, the use of water weeds as substitute feed could facilitate the development of a new type of fishery production system [26].

Azolla (mosquito fern), is a genus with seven species found in ponds, ditches, and wetlands throughout the world, from temperate to tropical regions. This aquatic plant is one of the fastest growing plants capable of doubling its biomass every 5–6 days [24]. It is considered to be a promising feed because of its high nutritive value, ease of cultivation, and high productivity [25, 39]. Azolla appears to be a good source of protein and contains almost all the essential amino acids, making it superior to wheat bran, maize and offal [12,17]. It also contains some probiotics biopolymers and naturally rich in minerals, such as iron, calcium, magnesium, potassium, phosphorus, and manganese, together with appreciable quantities of vitamin A, precursor beta-carotene, and vitamin B12 [8]. No

ISSN- 2394-5125 Vol 8, Issue 1, 2021

studies have been reported the effect of *Azolla filiculoides* as antioxidant on tilapia. Most of studies only test its effect on growth and as protein replacement. Incorporation of Azolla meal in the diet at 20% shows no adverse effect on growth of tilapia, which corresponds approximately to a reduction of 30% of fish meal protein on a control diet and it did not also affect significantly the fish carcass [4]. Consequently, this study will find out if the antioxidant properties of *A. filiculoides* could also stimulate the antioxidant capacity and metabolic response of Nile tilapia under stress environment.

Under normal conditions, the generated reactive oxygen species (ROS) are detoxified by the antioxidants present in the body and the generated ROS and the present antioxidants are in equilibrium. However, due to ROS overproduction or inadequate antioxidant defense, this equilibrium is hampered favoring the increase of ROS that leads to oxidative stress. Physico-chemical parameters and metabolic changes can induce distinct responses in fish antioxidant defense and fish exposed to hypoxia or hyperoxia showed marked antioxidant defense alterations [22]. The antioxidant capacity can be assessed by measuring plasma antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD, GPx and GR involves in protective mechanisms within tissue injury following oxidative process and phagocytosis [9], protects cells from excessive levels of hydrogen peroxide (H_2O_2) and intracellular lipid peroxides [41] and catalyzes the reduction of glutathione to yield reduced glutathione which is readily oxidized by ROS [45], respectively. On the other hand, for metabolic response, it can be assessed by measuring the plasma glucose (Gluc), triglycerides (Trig) and lactate (Lac). These served as indicator to stress [11] and their responses are considered adaptive and important for the fish to regain homeostasis [29].

Thus, it will be beneficial to tilapia farming if azolla could not only be an alternative cheap protein source but also as potential antioxidant that could enhance the resistance of tilapia under different stressors present in the environment. Therefore, this study aims to evaluate the rearing performance, antioxidant enzyme activities and metabolic response of Nile tilapia fed diet with different levels of *A. filiculoides* following thermal stress after ten weeks of rearing.

II. MATERIALS AND METHOD

2.1. Experimental plant and its extraction

Azolla was collected from Provincial Institute of Fisheries, Roxas, Isabela and washed with tap water to remove dirt and other debris. It was air-dried at room temperature for 3 days then oven-dried for 8hrs at 60°C and pulverized into fine powder form using pulverizer. The powdered azolla were packed in an airtight zipper bag and kept at 4 °C for further analysis.

The extraction procedures were performed with some modifications. The 1g of dried plant leaf powder was mixed with 10ml of 80% ethanol solvent and kept in an automatic mixer (HulamixerTM) for 24-48h. The sample was then centrifuged (Allegra X-12r centrifuge) at 7000rpm for 20mins and the supernatant was collected. After the collection of the supernatant the solvent was evaporated using a rotary evaporator (IKA RV10 Basis, HB10 Digital), and the extract was freeze-dried (Firstek Model FDA 4.5/-5.0) and stored at 4°C until further analysis [31].

2.2. Antioxidant assays of *Azolla filiculoides*

Antioxidant assays was conducted to evaluate the total antioxidant status (TAS) and Diphenyl-2-picrylhydrazyl (DPPH). Antioxidant content of azolla extract is presented in Table 1.

Antioxidant activity was measured using Randox® assay kit TAS. 2,2' –Azino-di-[3-ethylbenzthiazoline suphonate] (ABTS®) is the active reagent. Briefly, for the solutions 20µL of samples, blank and standard were added and separately mixed with 1ml chromogen. After mixing, the solutions were brought to room temperature and 200µL of each one was loaded into the 96-well plate and measured spectrophotometrically at 600nm for the initial absorbance (A1). After the first reading, the solutions on the 96-well plate were mixed again with 40µL and the absorbance was measured spectrophotometrically at 600nm after exactly 3mins (A2). Results were expressed as mmol/l. The antioxidant activity was measured using the following equations:

$$\Delta A \text{ of sample/standard/blank} = A2 - A1$$

$$\text{Factor} = \frac{\text{Concentrations of standard(provided by Randox)}}{(\Delta A \text{ blank} - \Delta A \text{ standard})}$$

$$\text{Mmol} = \text{Factor x } (\Delta A \text{ Blank} - \Delta A \text{ Sample}$$

ISSN- 2394-5125 Vol 8, Issue 1, 2021

The free radical scavenging activity was measured by using DPPH reagent with some modifications. Briefly, 100µl (1mg/ml concentration) of plant leaf extract was added to 100µl methanolic solution of DPPH (0.1 mM). The mixture was left to stand at room temperature for 30min in darkness. The absorbance of the resulting solution was then measured spectrophotometrically at 517nm [46]. The ability to scavenge the DPPH radical was calculated using the following equation described by Molyneoux [28].

$$\text{Scavenging, quenching or inhibition effect \% } = Q = \frac{(A_0 - Ac)}{A_0} \times 100$$

Where A_0 is the absorbance of the control (DPPH alone) and AC is the value for the sample with DPPH. Ascorbic acid was used as standard.

Table 1. TAS and DPPH of dried *Azolla filiculoides* extract.

Antioxidant parameters	Azolla Extract
Total Antioxidant Status (TAS)	1.13 mmol/L
Diphenyl-2-picrylhydrazyl (DPPH)	52.61 %

2.3. Fermentation of *Azolla filiculoides*

The leaves of azolla were air-dried. The dried leaves were finely ground and passed through a fine meshed sieve to ensure homogeneity. The fermentation process was adopted from [20] with some modifications. Briefly, the 1 kg of each aquatic plants was mixed with 200 ml molasses and 2ml of 10^5 cfu/g *Bacillus subtilis* in a plastic container covered with black trash bag and store in a cool dry room. The juice of fermented aquatic plants was then collected after 15 days of fermentation.

2.4. Proximate analysis of raw and fermented *Azolla filiculoides*

The proximate composition of raw and fermented azolla is presented in Table 2. It was analyzed at Regional Feed Chemical Analysis Laboratory, Tuguegarao City, Cagayan. Crude protein was analyzed by using the method foss tecator, crude fiber & fat by filter bag technique and moisture & ash by using gravimetric.

Table 2. Proximate analysis of raw and fermented *Azolla filiculoides*.

Proximate analysis (%)	Raw Azolla	Fermented Azolla[2]
Crude protein	24.5	26.7
Crude fiber	14.10	6.56
Crude lipid	2.19	1.81
Moisture	13.70	17.50
Ash	19.28	13.70

2.5. Diet preparation

Fermented and unfermented or raw leaf meal were used in the formulation of test diets. Two sets of experimental diets (Table 3) were formulated using raw and fermented azolla at 10, 20, 30, and 40% levels and a diet without azolla was used as the Control (C). All the diets were prepared in pelleted form using cornstarch as a binder. The experimental diets were oven-dried at 35 °C overnight and stored in a dry plastic container at 4ºC.

Table 3. Ingredients of the experimental diets with different levels of raw and fermented *A. filiculoides.*

	C[1]	Raw azolla[2]				Fermented azolla[3]			
Diet notation	C	10RA	20RA	30RA	40RA	10FA	20FA	30FA	40FA
Aquatic plants	0	10%	20%	30%	40%	10%	20%	30%	40%

ISSN- 2394-5125 Vol 8, Issue 1, 2021

inclusion rate									
Ingredients (g)									
Fish meal	300	300	300	300	300	300	300	300	300
Soy bean meal (SBM)	280	220	150	80	20	220	150	80	20
Corn bran	100	100	100	100	100	100	100	100	100
Corn starch	250	210	180	150	110	210	180	150	110
Aquatic plants	0	100	200	300	400	100	200	300	400
Soybean oil	50	50	50	50	50	50	50	50	50
Vitamins	10	10	10	10	10	10	10	10	10
Minerals	10	10	10	10	10	10	10	10	10
Proximate analysis (%)									
Crude protein	30.27	30.45	30.25	30.06	30.24	30.67	30.70	30.73	31.13
Crude lipid	13.05	12.00	10.75	9.50	8.46	11.97	10.67	9.38	8.30
Crude fibre	1.68	2.91	4.12	5.34	6.57	2.15	2.62	3.09	3.57
Ash	8.79	10.36	11.88	13.39	14.96	9.62	10.40	11.18	12.01
Dry matter	91.67	91.19	90.63	90.07	89.57	90.81	89.87	88.93	88.05

[1]C: Control-basal diet.
[2]Raw azolla: 10RA -100 g/kg raw azolla, 20RA - 200 g/kg raw azolla, 30RA - 300 g/kg azolla and 40RA - 400 g/kg azolla.
[3]Fermented azolla: 10FA -100 g/kg fermented azolla, 20FA - 200 g/kg fermented azolla, 30FA - 300 g/kg fermented azolla and 40FA - 400 g/kg fermented azolla.

2.6. Fish rearing, feeding and sampling

Fish were obtained from San Mateo, Isabela and transported to the fishpond of ISU-Echague in well oxygenated bags. They were acclimatized in hapa (2m x 4m x 1m) prior to the conduct of the experiment. Fish were randomly weighed and distributed with 25 fish in each of the in plastic tub. Feeding was done twice daily at 8% of their body weight at 0800 h and 1500 h. Each plastic tub was aerated. Feces and uneaten feed were siphoned-out and one-third of the water was replaced twice a week. Water quality parameters were monitored and kept within safe levels: dissolved oxygen (DO) 6-7 mg/L, temperature 25-30°C and pH 6.5-7.8.

2.7. Growth and survival

Fish were weighed every 2 weeks with a digital scale. The quantity of feed given was readjusted after each weight sampling and survival in each tank was monitored daily. Final weight (Wf), weight gain (WG) and specific growth rate (SGR) were used as indices for the growth performance of fish.

WG (%) = 100 x ((Wf – Wi)/Wi)
SGR (%) = 100 x ((ln(Wf) – ln(Wi))/T
where Wi is the initial body weight (g), Wf the final body weight (g), ln the natural logarithm and T the number of days in the feeding period.
FCR = feed intake / weight gain
% Survival = final / initial count *100

2.8. Thermal stress

This experiment was conducted to find out if different experimental diets could affect the survival, antioxidant enzyme activities and metabolic response of tilapia under thermal stress condition. After 10-week rearing, 15 fish from each treatment were randomly selected and immediately transferred to one of the 10L plastic tubs in duplicates. Temperature regulator was installed in each of the plastic tubs to control 3 different temperatures (low: 25 °C, medium: 28 °C and high: 33 °C). Aerator was also installed in each of the plastic tubs. Mortality was recorded daily. Blood samples were taken after 10 weeks feeding of experimental diets and one day after thermal stress test.

2.9. Antioxidant capacity and metabolic response

Blood samples were taken after rearing. Sampled fish was quickly anesthetized with tricaine methane sulphonate (MS-222) at 100 mg•l^{-1}. Approximately 200 µl heparinized blood was withdrawn from the caudal vessel of 3 fish

ISSN- 2394-5125 Vol 8, Issue 1, 2021

per plastic tubs using 1-ml sterile syringe with 23 gauge needles. Heparinized blood was centrifuged for 5 min at 1800 g and the plasma was drawn and immediately frozen (-4^{o}C) for later evaluation of antioxidant capacity and metabolic response.

The antioxidant capacity was analyzed with enzyme linked immunosorbent assay (ELISA) reader for superoxide dismutase (SOD) and SP-830 plus metertech spectrophotometer for glutathione peroxidase (GPx) and glutathione reductase (GR). The volumes of plasma used was 10, 10 and 20 µl for SOD, GPx and GR analysis, respectively.

SOD activity was measured by its ability to inhibit superoxide radical dependent reactions. The reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase (80 U•l^{-1}, 250 µl), superoxide and uric acid were produced from xanthine. The superoxide radical was then reacted with INT to produce a red formazan dye. The optical density was measured at 505 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings at 30s and 3min after adding xanthine oxidase. A reference standard SOD was supplied with the Randox Kit (Crumlin, Co. Antrim, UK). One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50% [14]. One unit of activity was expressed in U•ml^{-1}.

GPx activity was measured based on the method described by Paglia et al., [37]. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized form of glutathione was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm were measured. Briefly, 15 µl diluted plasma mixture was added to the reaction mixture containing 40 µl cumene hydroperoxide and 10 mM buffer. The optical density of NADPH was measured at 340 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings at the first 3 min after adding cumene hydroperoxide. One unit of activity was expressed in U•ml^{-1}.

Finally, GR catalyses the reduction of glutathione in the presence of NADPH, which is oxidized to NADP+. The decrease in absorbance at 340 nm was measured. This assay was carried out using Randox laboratories kit according to manufacturer's instructions [14]. One unit of activity was expressed in U•ml^{-1}.

ELISA reader with individual Randox kits were used for determination of Gluc (Randox, GOD-PAP), Trig (Randox-GPO-PAP) and Lac (Randox, PAP). Methods was adapted to a 96-well plate using 3 µl samples and 300 enzyme reagents [38]. Gluc, Trig and Lac levels were expressed in mg•dl^{-1} plasma.

2.10. Statistical analysis

An arcsine transformation was used before processing percentage data. Levene and Kolmogorov–Smirnov's test for homogeneity of variance and normality were applied on the data of growth, survival, antioxidant capacity and metabolic response. One-way analysis of variance (ANOVA) was performed to find out the effects of treatments on rearing performance, antioxidant capacity (SOD, GPx and GR) and metabolic response (Gluc, Trig and Lac). Two-way ANOVA was carried out to find out the main effect of preparation (Raw and Fermented Azolla) and levels (10, 20, 30 and 40%). Tukey's test was carried out to compare differences between levels of each factor. The significant level applied was 5% and SAS v.9 software was used in all the analysis.

III. RESULTS

3.1. Rearing Performance

3.1.1. Raw *Azolla filiculoides*

RA had significant effects on growth performance of Nile tilapia after ten weeks of rearing (Table 4). Wf of 10RA, 20RA and 30RA were significantly higher as compared to C but comparable to that of 40RA. In addition, WG and SGR of 20RA showed no significant difference to that of 10RA, 30RA and 40RA but significantly higher to C. No significant difference was observed on FCR and %Survival among all treatments after ten weeks of rearing.

Table 4. **Rearing performance of *O. niloticus* fed diet with different levels of raw *A. filiculoides* reared for 10 weeks.**

Treatment	Wi (g)	Wf (g)	WG (%)[1]	FCR[2]	SGR (%)[3]	%Survival[4]
C	1.50 [a]	18.05[b]	1109.5 [b]	2.45 [a]	3.56 [b]	74.00 [a]

ISSN- 2394-5125 Vol 8, Issue 1, 2021

	(0.10)	(0.15)	(91.35)	(0.05)	(0.11)	(2.00)
10RA	1.45 [a] (0.05)	24.10 [a] (1.30)	1552.30 [ab] (108.60)	1.95 [a] (0.35)	4.00 [ab] (0.09)	74.00 [a] (2.00)
20RA	1.45 [a] (0.35)	24.75 [a] (2.35)	1652.30[a] (246.00)	1.85 [a] (0.05)	4.08 [a] (0.20)	74.00 [a] (2.00)
30RA	1.40 [a] (0.10)	23.25 [a] (0.85)	1539.70 [ab] (56.10)	2.05 [a] (0.05)	3.99 [ab] (0.04)	74.00 [a] (2.00)
40RA	1.45 [a] (0.15)	20.30 [ab] (1.10)	1336.80 [ab] (63.20)	2.00 [a] (0.20)	3.80 [ab] (0.06)	74.00 [a] (2.00)

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1] WG (%) = (Wf-Wi)/Wi) x 100.
[2] FCR = feed intake / weight gain.
[3] SGR (%) = (ln mean final weight) – (ln mean initial weight)/ no. of days) x 100.
[4] Survival (%) = final count/initial count x 100.

3.1.2. Fermented *Azolla filiculoides.*

FA had significant effects on growth performance of Nile tilapia after 10 weeks of rearing (Table 5). Wf of 10FA, 20FA, 30FA and 40FA were significantly higher as compared to C. In addition, WG of 20FA and 30FA were comparable to that of 10FA and 40FA but significantly higher to C. Moreover, SGR of 20FA, 30FA, and 40FA had no significant difference to 10FA but significantly higher to that of C. FCR of C was the highest among treatments. However, no significant difference was found on %Survival among all treatments after 10 weeks of rearing.

Table 5. Rearing performance of *O. niloticus* fed diet with different levels of fermented *A. filiculoides* reared for 10 weeks.

Treatment	Wi (g)	Wf (g)	WG (%)[1]	FCR[2]	SGR (%)[3]	%Survival[4]
C	1.50 [a] (0.10)	18.05 [b] (0.15)	1109.5 [b] (91.35)	2.45 [a] (0.05)	3.56 [b] (0.11)	60.0 [a] (0.00)
10FA	1.50[a] (0.10)	23.85[a] (0.35)	1472.5[ab] (62.10)	2.05 [b] (0.15)	3.93[ab] (0.05)	72.00[a] (2.00)
20FA	1.40 [a] (0.00)	26.95 [a] (0. 85)	1884.50 [a] (62.15)	2.10 [b] (0.10)	4.26 [a] (0.04)	74.00 [a] (2.00)
30FA	1.55 [a] (0. 05)	25.55 [a] (1. 05)	1668.00 [a] (121.15)	2.00 [b] (0.00)	4.10 [a] (0.04)	72.00 [a] (4. 00)
40FA	1.40 [a] (0.00)	24.70 [a] (1.00)	1558.00 [ab] (46.15)	2.10 [b] (0. 10)	4.01 [a] (0. 10)	70.00 [a] (2.00)

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1] WG (%) = (Wf-Wi)/Wi) x 100.
[2] FCR = feed intake / weight gain.
[3] SGR (%) = (ln mean final weight) – (ln mean initial weight)/ no. of days) x 100.
[4] Survival (%) = final count/initial count x 100.

3.1.3. Two-way analysis and interaction effect on growth and survival.

Disregarding types of azolla preparation, Wf of fish fed diet with 10%, 20% and 30% was significantly higher as compared to that of 40% and C (Table 6). In addition, WG of 20% was significantly higher as compared to C. SGR of 10%, 20%, 30% and 40% were also significantly higher as compared to that of C. However, no significant difference was found on FCR and %Survival among all treatments after 10 weeks of rearing.

Disregarding levels, fish fed with RA and FA had no significant difference in growth parameters. However, fish fed diet with FA had higher % Survival after 10 weeks of rearing.

Table 6. Mean rearing performance of *O. niloticus* fed diet with different levels of raw and fermented *A. filiculoides* for 10 weeks of rearing.

Parameter[1]	Levels[1]	Types[2]	Pr>F	SEM[3]

ISSN- 2394-5125 Vol 8, Issue 1, 2021

	0	10	20	30	40	RA	FA		
Wi	1.50[a] (0.10)	1.48[a] (0.04)	1.43[a] (0.14)	1.48[a] (0.06)	1.43[a] (0.06)	1.44[a] (0.07)	1.46[a] (4.22)	0.9947	4.0×10^{-2}
Wf	18.05[b] (0.15)	23.98[a] (0.55)	25.85[a] (1.20)	24.40[a] (0.86)	22.50[b] (1.40)	23.10[a] (0.86)	25.26[a] (0.54)	0.0096	1.0×10^{1}
WG	1109.5[b] (91.35)	1512.4[ab] (56.02)	1768.3[a] (124.01)	1548.8[ab] (30.12)	1502.4[ab] (110.16)	1520.2[a] (68.87)	1645.7[a] (65.38)	0.0318	3.3×10^{4}
SGR	3.56[b] (0.11)	3.97[a] (0.04)	4.17[a] (0.10)	4.00[a] (0.02)	3.95[a] (0.09)	3.97[a] (0.05)	4.08[a] (0.05)	0.0048	1.9×10^{-2}
FCR	2.45[a] (0.05)	2.00[a] (0.15)	1.93[a] (0.05)	2.08[a] (0.05)	1.98[a] (0.09)	2.00[a] (0.0)	2.13[a] (0.04)	0.3188	1.3×10^{-2}
Survival%	67.00[a] (4.12)	72.00[a] (1.63)	74.00[a] (2.00)	73.00[a] (01.91)	73.00[a] (1.91)	68.00[b] (2.19)	74.00[a] (0.75)	0.050	1.2×10^{1}

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1] Levels used in the experiment: 0 (Control); 10 (100 g/kg); 20 (200 g/kg); 30 (300 g/kg); 40 (400 g/kg).
[2]Types: R - raw azolla; F - fermented azolla.
[3]Standard error of mean.

3.2. Antioxidant capacity

One-way and two-way ANOVA (levels and types) revealed that SOD, GPx and GR activity had no significant difference among treatments after 10 weeks of rearing (Table 7, Table 8 and Table 9).

Table 7. Mean activities of plasma antioxidant capacity of *O. niloticus* after fed diet with different levels of raw *A. filiculoides* for 10 weeks of rearing.

Treatment	Antioxidant capacity[1]		
	SOD	GPx	GR
C	0.28[a] (0.05)	1.19[a] (0.27)	0.039[a] (0.004)
10RA	0.26[a] (0.02)	1.32[a] (0.07)	0.040[a] (0.005)
20RA	0.23[a] (0.02)	1.43[a] (0.09)	0.042[a] (0.008)
30RA	0.27[a] (0.03)	1.33[a] (0.10)	0.037[a] (0.003)
40RA	0.29[a] (0.03)	1.14[a] (0.19)	0.035[a] (0.002)

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Antioxidant parameters: SOD-Superoxidase dismutase, GPx-Glutathione peroxidase, GR- Glutathione reductase.

Table 8. Mean activities of plasma antioxidant capacity of *O. niloticus* after fed diet with different levels of fermented *A. filiculoides* 10 weeks of rearing

Treatment	Antioxidant capacity[1]		
	SOD	GPx	GR
C	0.28[a] (0.05)	1.19[a] (0.27)	0.039[a] (0.004)
10FA	0.25[a] (0.03)	1.36[a] (0.08)	0.040[a] (0.006)
20FA	0.22[a] (0.03)	1.47[a] (0.03)	0.046[a] (0.004)

ISSN- 2394-5125 Vol 8, Issue 1, 2021

30FA	0.25 [a] (0.05)	1.35 [a] (0.15)	0.039 [a] (0.004)
40FA	0.28 [a] (0.04)	1.22 [a] (0.11)	0.035 [a] (0.003)

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Antioxidant parameters: SOD - Superoxidase dismutase, GPx - Glutathione peroxidase, GR -Glutathione reductase.

Table 9. Mean activities of plasma antioxidant capacity of *O. niloticus* fed diet withdifferent levels of raw and fermented *A. filiculoides* for 10 weeks of rearing.

Parameters[1]	Levels[2]					Types[3]		Pr>F	SEM[4]
	0	10	20	30	40	R	F		
SOD	0.28[a] (0.04)	0.26[a] (0.01)	0.23[a] (0.01)	0.26[a] (0.02)	0.28[a] (0.02)	0.26[a] (0.01)	0.25[a] (0.01)	0.8578	2.1×10^{-3}
GPx	1.19[a] (0.27)	1.34[a] (0.04)	1.45[a] (0.04)	1.38[a] (0.07)	1.18[a] (0.08)	1.30[a] (0.05)	1.35[a] (0.05)	0.7315	3.8×10^{-2}
GR	0.039[a] (0.004)	0.040[a] (0.003)	0.040[a] (0.003)	0.038[a] (0.001)	0.035[a] (0.001)	0.039[a] (0.002)	0.040[a] (0.002)	0.7905	4.2×10^{-5}

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Antioxidant parameters: SOD-Superoxide dismutase; GPx-Glutathione peroxidase; GR-Glutathione reductase
[2]Levels used in the experiment: 0 (Control); 10 -100g/kg; 20 -200g/kg; 30–300g/kg; 40-400g/kg.
[3]Types: R - raw azolla; F - fermented azolla.
[4]Standard error of mean.

3.3. Metabolic response

One-way and two-way ANOVA (levels and types) of treatments in Gluc, Trigs and Lac activity had no significant difference among treatments after 10 weeks of rearing (Table 10, Table 11 and Table 12).

Table 10. Mean activities of plasma metabolic response of *O. niloticus* after fed diet with different levels of raw *A. filiculoides* for 10 weeks of rearing.

Treatment	Metabolic response[1]		
	Gluc	Trigs	Lac
C	48.00[a] (6.00)	121.00[a] (6.00)	35.50[a] (4.50)
10RA	42.50[a] (3.50)	124.50[a] (6.50)	36.00[a] (4.00)
20RA	44.00[a] (6.00)	118.50[a] (2.50)	35.00[a] (2.00)
30RA	52.50[a] (7.50)	124.00[a] (4.00)	34.00[a] (2.00)
40RA	52.00[a] (9.00)	126.50[a] (10.50)	36.50[a] (2.50)

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Metabolic parameters: Gluc – Glucose; Trigs – Triglycerides; Lac – Lactate.

Table 11. Mean activities of plasma metabolic response of *O. niloticus* after fed diet with different levels of fermented *A. filiculoides* for 10 weeks of rearing.

Treatment	Metabolic response[1]

ISSN- 2394-5125 Vol 8, Issue 1, 2021

	Gluc	Trig	Lac
C	48.00 [a] (6.00)	121.00 [a] (6.00)	35.50 [a] (4.50)
10FA	44.50 [a] (7.50)	121.50 [a] (3.50)	34.50 [a] (2.50)
20FA	41.50 [a] (5.50)	117.00 [a] (3.00)	32.50 [a] (0.50)
30FA	40.50 [a] (8.50)	119.50 [a] (4.50)	33.50 [a] (1.50)
40FA	50.00 [a] (9.00)	124.00 [a] (8.00)	36.00 [a] (2.00)

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Metabolic parameters: Gluc – Glucose; Trigs – Triglycerides; Lac – Lactate.

Table 12. Mean activities of plasma metabolic response of *O. niloticus* fed diet with different levels of raw and fermented *A. filiculoides* for 10 weeks of rearing.

Parameters[1]	Levels[2]					Types[3]		Pr>F	SEM[4]
	0	10	20	30	40	R	F		
Gluc	48.00[a] (6.00)	43.50[a] (3.42)	42.75[a] (3.40)	46.50[a] (5.78)	48.00[a] (5.22)	47.75[a] (3.09)	44.13[a] (3.24)	0.8898	1.0×10^{-2}
Trigs	121.00[a] (6.00)	123.00[a] (3.13)	117.75[a] (1.65)	121.75[a] (2.78)	125.25[a] (5.43)	123.38[a] (2.73)	120.50[a] (2.17)	0.9561	7.0×10^{1}
Lac	35.50[a] (4.50)	35.25[a] (1.97)	33.75[a] (0.10)	33.75[a] (0.03)	36.25[a] (1.31)	35.38[a] (1.10)	34.13[a] (0.83)	0.9685	1.4×10^{1}

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Metabolic parameters: Gluc – Glucose; Trigs – Triglycerides; Lac – Lactate.
[2]Levels used in the experiment: 0 (Control); 10-100 g/kg; 20-200 g/kg; 30–300 g/kg; 40-400 g/kg.
[3]Types: R - raw azolla; F - fermented azolla.
[4]Standard error of mean.

3.4. Thermal stress

Both factors showed (effect of different levels of raw and fermented azolla and different levels of temperature) no significant effect on antioxidant capacity and metabolic response one-day after thermal stress (Table 13 and Table 14).

Table 13. Mean activities of plasma antioxidant capacity and metabolic response of *O. niloticus* fed diet with different levels of raw *A. filiculoides* at 1- day after thermal stress

	Thermal stress[3]			Levels[4]					Pr>F	SEM
	Low	Medium	High	0	10	20	30	40		
Antioxidant capacity[1]										
SOD	0.31[a] (0.01)	0.30[a] (0.02)	0.31[a] (0.02)	0.31[a] (0.17)	0.30[a] (0.02)	0.29[a] (0.02)	0.31[a] (0.01)	0.32[a] (0.01)	0.9982	2.8×10^{-3}
GPx	1.38[a] (0.09)	1.28[a] (0.07)	1.37[a] (0.11)	1.31[a] (0.13)	1.35[a] (0.10)	1.31[a] (0.12)	1.35[a] (0.11)	1.36[a] (0.09)	1.0000	1.2×10^{-1}
GR	0.041[a] (0.001)	0.037[a] (0.001)	0.041[a] (0.001)	0.038[a] (0.002)	0.040[a] (0.001)	0.040[a] (0.001)	0.040[a] (0.001)	0.041[a] (0.002)	0.9887	3.5×10^{-5}

ISSN- 2394-5125 Vol 8, Issue 1, 2021

Metabolic response[2]										
Gluc	43.25[a] (3.75)	41.88[a] (2.97)	43.25[a] (3.02)	42.00[a] (2.98)	42.50[a] (3.19)	43.33[a] (4.32)	40.17[a] (3.53)	45.17[a] (4.02)	0.9999	$1.2x10^{2}$
Trig	135.50[a] (7.52)	122.88[a] (3.14)	137.88[a] (6.85)	136.17[a] (7.60)	132.67[a] (7.54)	131.33[a] (9.00)	130.33[a] (7.44)	134.00[a] (7.15)	0.9988	$5.2x10^{2}$
Lac	35.38[a] (0.98)	34.13[a] (0.93)	35.88[a] (0.85)	35.83[a] (1.74)	34. 33[a] (0.98)	35.50[a] (1.05)	35.67[a] (1.11)	35.00[a] (1.29)	0.9992	$1.4x10^{1}$

Means (±SE) without common superscript are significantly different ($p \leq 0.05$).
[1]Antioxidant parameters: SOD - Superoxidase dismutase, GPx - Glutathione peroxidase, GR - Glutathione reductase.
[2]Metabolic parameters: Gluc - Glucose, Trig - Triglycerides, Lac - Lactate.
[3]Thermal stress: Low - 25 ˚C, Medium - 28 ˚C, High - 33 ˚C.
[4]Levels used in the experiment: 0 (Control); 10 - 100 g/kg; 20 - 200 g/kg; 30 – 300 g/kg; 40 - 400 g/kg.

Table 14. Mean activities of plasma antioxidant capacity and metabolic response of *O. niloticus* fed diet with different levels of fermented *A. filiculoides* at 1-day after thermal stress

Parameters	Thermal stress[3]			Levels[4]					Pr>F	SEM
	Low	Medium	High	0	10	20	30	40		
Antioxidant capacity[1]										
SOD	0.30[a] (0.01)	0.30[a] (0.01)	0.32[a] (0.01)	0.31[a] (0.01)	0.29[a] (0.01)	0.32[a] (0.02)	0.30[a] (0.01)	0.31[a] (0.01)	0.9911	$2.5x10^{-3}$
GPx	1.36[a] (0.10)	1.30[a] (0.09)	1.38[a] (0.09)	1.31[a] (0.12)	1.39[a] (0.11)	1.32[a] (0.12)	1.30[a] (0.10)	1.37[a] (0.10)	1.000	$1.2x10^{-1}$
GR	0.042[a] (0.001)	0.038[a] (0.002)	0.041[a] (0.002)	0.038[a] (0.002)	0.040[a] (0.002)	0.040[a] (0.002)	0.038[a] (0.002)	0.040[a] (0.002)	0.5080	$5.9x10^{-3}$
Metabolic response[2]										
Gluc	43.25[a] (3.75)	42.25[a] (3.17)	41.75[a] (3.09)	42.00[a] (2.98)	40.33[a] (3.46)	45.33[a] (4.63)	40.83[a] (3.19)	43.17[a] (4.04)	1.0000	$1.3x10^{2}$
Trig	139.38[a] (7.20)	123.63[a] (2.60)	135.50[a] (7.51)	136.17[a] (7.60)	134.67[a] (6.89)	132.17[a] (8.87)	131.00[a] (8.22)	133.50[a] (7.44)	0.9984	$5.2x10^{2}$
Lac	35.38[a] (0.98)	33.88[a] (1.00)	36.50[a] (0.89)	35.83[a] (1.74)	35.00[a] (1.12)	35.33[a] (1.05)	35.00[a] (1.39)	34.83[a] (1.19)	0.9999	$1.6x10^{1}$

[1]Antioxidant parameters: SOD - Superoxidase dismutase, GPx - Glutathione peroxidase, GR - Glutathione reductase.
[2]Metabolic parameters: Gluc - Glucose, Trig - Triglycerides, Lac - Lactate.
[3]Thermal stress: Low - 25 ˚C, Medium - 28 ˚C, High - 33 ˚C.
[4]Levels used in the experiment: 0 (Control); 10 - 100 g/kg; 20 - 200 g/kg; 30 – 300 g/kg; 40 - 400 g/kg.

IV. DISCUSSION

Azolla as feed ingredient could enhance growth performance of fish. In this study, the Wf of 10RA, 20RA, 30RA and 40RA were increased by 34%, 37%, 29% and 13% as compared to C, respectively. In addition, the WG of 10RA, 20RA, 30RA and 40RA, were increased by 40%, 49%, 39% and 20% as compared to C, respectively. The improvement on growth could be attributed to the nutrients present in azolla. These aquatic fern has a substantial amount of protein (20–30% dry weight); vitamins, especially retinol and several B vitamins; b-carotene [23,3] and minerals such as Ca, P, K, Fe, Cu and Mg, which could be found in suffice amount [42,5]. Studies also reported that the crude extract of Azolla spp. contains various antioxidant, like phyto-constituents such as, tannins, phenolic contents and flavonoids [36]. Many of herbivorous and omnivorous fish such as *Labeo rohita*, *Labeo fimbriatus*, *Labeo calbasu*, *Cirrhinus mrigala*, *Barbonymus gonionotus* and *Tilapia zillii* have been reported that utilize azolla as feed ingredients [34].

ISSN- 2394-5125 Vol 8, Issue 1, 2021

Optimum level of raw azolla helps to enhance the growth performance of fish. Our result showed that, 10-40RA could be used in the diet of *O. niloticus*. However, among the levels, 20RA had better effect in fish growth performance. It has the highest increased value of 37%, 39% and 15% in Wf, WG and SGR among levels as compared to C, respectively. In other study, *Azolla pinnata* the other species, has been recommended that 20% inclusion level for *Cyprinus carpio* which showed no adverse effect on digestibility [21]. Better economic performance was also found in *O. niloticus* at 20% level of inclusion [3]. In addition, 25% inclusion of *A. pinnata* improved feed intake, protein productive value (PPV), FCR and protein efficiency ratio (PER) in *Tilapia zillii* [1]. Moreover, *A. filiculoides* at 30%, showed no adverse impact on nutrient digestibility and nutritional quality of the fish [4], and at 31.8% inclusion of *Azolla nilotica*, shows no harmful impact on survivability, growth performance, utilization of feed and economical parameters [14]. Highest WG and SGR and lowest FCR was also observed in *O. niloticus* at 15% inclusion of *A. pinnata* [18]. Therefore, azolla can be considered as nutritional source of feed for herbivorous-omnivorous fish [19].

Optimum level of fermented azolla helps to enhance the growth performance of animals. In this study, the Wf of 10FA, 20FA, 30FA and 40FA were increased by 32%, 49%, 42% and 37% as compared to C, respectively. In addition, the WG of 10FA, 20FA, 30FA and 40FA, were increased by 33%, 70%, 50% and 40% as compared to C, respectively. Our results are in agreement with Mosha [32], who reported that azolla at 10-45% inclusion level can be incorporated in the diet for Tilapia species, except for *T. zillii* which requires more than 40% protein content. 20FA had the highest increased value of 49%, 70% and 20 in Wf, WG and SGR among the levels as compared to C, respectively. Our result is similar to Hundare et al. [20], who reported that *O. niloticus* fed with 20-30FA showed better growth (length gain, WG, SGR) and FCR after 60 days of rearing. Aside from the nutritional components of azolla, the improvement on growth could be attributed to the fermentation process which reduces the anti-nutritional factors such as tannins and oxalates present in raw aquatic plants [16]. Fermentation increased the levels of protein, especially amino acids. During the fermentation process, there is an increase in the amount of nitrogen, and the increase is caused by the activity of protease enzymes that break down proteins, such that they are more soluble in water. Fermentation process was not only able to reduce the high crude fiber content, but could also increase the nutritional value of the feed ingredients [36]. It increases the number of microorganisms that intensify the metabolism in feed, thereby producing new feed products [35].

Fish performance continued to deteriorate with increasing dietary level of azolla. This reduction was extremely sharp when dry and fresh azolla was used as total diets [15]. The deplete in growth parameters of *O. niloticus* observed with 40RA could be attributed with high amounts of indigestible fiber, carbohydrates, and some anti-nutritional factors (phenols, tannins, phytatess and saponin) and insufficient essential amino acids [10]. In other study, inclusion level of more than 20% of azolla resulted also on lowering the growth performane in *O. niloticus* [40]. Result on growth performance in this study is in contrast with those obtained with [1] who stated that *O. niloticus* fed with *A. pinnata* grows well at levels up to 42% of inclusion level in diets with 35% crude protein.

Under stressful conditions, glutathione related enzymes were gradually decreased due to its antioxidant mechanisms [30]. It has been reported that gradual increased in activities of antioxidant enzymes, which could be an effective response for the organism to neutralize free radical scavenging and prevent the induction of oxidative stress, which might lead to disease or even death [15]. As temperature rises above their optimal limit, their survival rate will be off-set due to alteration in the metabolic activity [43]. Freshwater fish species has the ability to adjust their thermal sensitivity but was limited [35]. Therefore, understanding the mechanism underlying the physiological responses in freshwater fishes is key to predicting the effects of climate change on fish population [33]. In addition, the simultaneous stress by temperature change could accelerate the alteration in the antioxidant enzymes activities of tilapia [27]. Fish can be compromised under temperature stress because of thermal impairment of protein function [2]. The different inclusion levels of azolla could enhance antioxidant capacity (SOD, GPx, GR) and metabolic response (Gluc, Trig, Lac) of animals in a certain period of time. However, no significant difference was found among activities of antioxidant capacity and metabolic response of Nile tilapia fed diet with raw and fermented azolla. Temperature stress may not be enough to stimulate the antioxidant capacity and metabolic response since 25 to 33°C is near to optimum temperature management for tilapia and it could be because tilapia can tolerate a wide range of temperature.

V. CONCLUSION

ISSN- 2394-5125 Vol 8, Issue 1, 2021

Overall, these results indicated that RA and FA at different inclusion levels enhance rearing performance of Nile tilapia. The inclusion of 20RA and 20-30FD in the diet could be a potential feed ingredient in the diet of Nile tilapia.

VI. ACKNOWLEDGEMENTS

This study acknowledged the funding support of the Department of Science and Technology – Philippine Council for Agriculture, Aquatic Resources Research and Development (DOST-PCAARRD). We also acknowledge the Ministry of Science and Technology (MOST), Taiwan for the scholarship to the first author.

VII. REFERENCES

[1] Abdel-Tawwab M. The preference of the omnivorous macrophages, *Tilapia zillii* (Gervais) to consume a natural free floating fern, *Azolla pinnata*. J World Aquacult Soc 2008;39; 104–112.
[2] Abele D, Puntarulo S. Formation of reactive species and induction of antioxidant defense systems in polar and temperate marine invertebrates and fish. Comp Biochem Physiol 2004;138: 405-415.
[3] Abou Y, Fiogbe ED, Micha JC. A Preliminary Assessment of Growth and Production of Nile Tilapia, *Oreochromis niloticus* (L) fed Azolla Based Diets in Earthen Ponds. J Appl Aquacult 2007 19, 55–69.
[4] Abou Y, Fiogbe ED, Beckers Y, Micha JC. Approximate compositional values and tissue fatty acid profiles of nile tilapia (*Oreochromis niloticus* L.) Fed azolla-diets in earthen ponds. Food and Nutri Sci 2011;2: 964-973.
[5] Anitha KC, Rajeshwari YB, Prasanna SB, Shree JS. Nutritive evaluation of Azolla as livestock feed. J Exp Biol Agricult Sci 2016;4: 670–674.
[6] Antoine T, Wery P, Micha JC Van Hove C. Comparison of the growth and chemical composition of *Oreochromis niloticus* (Tilapia) and *Cichlasoma melanturum* (Theraps) fed with Azolla. Aquaculture 1987;66, 181–196.
[7] Bakke AM, Krogdahl A, Penn M, Refstie S, Penn M. Important antinutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. Aquacult Res 2010;41(3):333 – 344.
[8] Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis 1991; 1:3-26.
[9] Basak B, Pramanik MAH, Rahman MS, Tarafdar SU, Roy BC. Azolla (Azolla pinnata) as a feed ingredient in broiler ration. Int J Poult Sci 2002;1: 29–34.
[10] Biagini G, Sala D, Zini I. Diethyldithiocarbomate, a superoxide dismutase inhibitor, counteracts the maturation of ischemic-like lesions caused by endothelin-1 intrastriatal injection. Neurosci Lett 1995;190: 212-216.
[11] Brucka JE, Kawczuga D. Antioxidant status and lipid peroxidation in blood of common carp *Cyprinus carpio*. Polish J of Environ Stud 2011;20(3): 541-550.
[12] Chainy GBN, Paital B Dandapat J. An overview of seasonal changes in oxidative stress and antioxidant defense parameters in some invertebrate and vertebrate species. Hindawi Publishing Corporation Scientifica 2016; Article ID 6126570: 1-8.
[13] Cherry lDM, Prasad RMV, Jagadeeswara, Rao S, Jayalaxmi P, Srinivas KD. A study on the nutritive value of *Azolla pinnata*. Livesst Res Int 2014;2: 13–15.
[14] Ebrahim MSM, Zeinhom MM, Abou-Seif RA. Response of Nile tilapia (*Oreochromis niloticus*) fingerlings to diets containing Azolla meal as a source of protein. J Arabian Aquacult Soc 2007;2: 54–69.
[15] El-Sayed AFM. Effects of substituting fish meal with *Azolla pinnata* in practical diets for fingerlings and adult Nile tilapia, *Oreochromis niloticus* (L). Aquacult Fish Manage 1992;23: 167-173.
[16] Francis G, Makkar HPS, Becker K. Antinutritional factors present in plant derived alternate fish feed ingredients and their effects in fish. Aquaculture 2001;199: 197- 227. Free Radical Biol Med 27:951–965.
[17] Francis G, Makkar HPS, and Becker K. Effects of cyclic and regular feeding of *Quillaja* saponin supplemented diet on growth and metabolism of common carp (*Cyprinus carpio* L.). Fish Physiol Biochem 2002b;24: 343-350.
[18] Fiogbe ED, Micha JC, Van Hove C. Use of a natural aquatic fern, *Azolla microphylla*, as a main component in food for the omnivorous-phytoplanktonophagous tilapia, *Oreochromis niloticus* L. J Appl Ichthyol 2004; 20: 517–520.
[19] Hossiny H, Setoudeh M, Rokni H, Dehghanzadeh H, Cheraghcheshm M. Using of silage Azolla in Guilan male calves nutrition. Proceedings of Third National Congress of Recycling and Reuse of Reneweable Organic Resources in Agriculture Islamic Azad University; 2008.

ISSN- 2394-5125 Vol 8, Issue 1, 2021

[20] Hundare SK, Pathan DI, Ranadive AB. Use of Fermented Azolla in Diet of Tilapia Fry (*Oreochromis niloticus*). Int J of Bioresour Stress Mgt 2018;9(6):702-706.
[21] Gangadhar B, Sridhar N, Saurabh S, Raghavendra CH, Hemaprasanth KP, Raghunath MR et al. Growth response of *Cirrhinus mrigala* fry to Azolla (*Azolla pinnata*) incorporated diets. Fish Technol 2014;51: 156–161.
[22] Guerriero G, Di Finizio A, Ciarcia G. Stress-induced changes of plasma antioxidants in aquaculture sea bass, *Dicentrarchus labrax*. Comp Biochem Physiol Part A 2002;132, 205-211.
[23] Leonard V. Use of Aquatic Fern (*Azolla filiculoides*) in Two Species of Tropical Fish (*Oreochromis niloticus* and *Tilapia rendalli*). PhD Thesis 1997. Presses Universities the Namur, Belgium, 276.
[24] Leonard V, Breyne C, Micha JC, Larondelle Y. Digestibility and transit time of *Azolla filliculoides* Lamarck in *Oreochromis aureus* (Steindachner). Aqua Res 1998;29: 159-165.
[25] Maity J, Patra BC. Effect of replacement of fishmeal by Azolla leaf meal on growth, food utilization, pancreatic protease activity and RNA/DNA ratio in the fingerlings of *Labeo rohita* (Ham.). Can J Pure Appl Sci 2008; 323.
[26] Magouz FI, Mahmoud AO, Dawood, Mahmoud FI, Salem, Ayman AI, Mohamed. The effects of fish feed supplemented with Azolla meal on the growth performance, digestive enzyme activity, and health condition of genetically-improved farmed tilapia (*Oreochromis niloticus*). Sciendo Ann Anim. Sci Vol. 20, No. 3 2020;1029–1045.
[27] Min EY, Jeong JW, Kang JC. Thermal effects on antioxidant enzymes response in Tilapia, *Oreochromis niloticus* exposed Arsenic. J Fish Pathol 2014;27(2): 115-125.
[28] Molyneoux P. The use of the stable radical Diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol 2004;26(2) : 211-21.
[29] Mommsen TP, Vijayan MM, Moon TW. Cortisol in teleosts: dynamics, mechanisms olfaction, and metabolic regulation. Rev Fish Biol Fish 1999;9: 211-268.
[30] Mones GH, Angeles IP Jr. Performance of Red Tilapia (*Oreochromis sp*.) Fed Diet with Fermented Banana (*Musa Acuminata* × *Balbisiana*) Peel at Different Stages of Ripeness Following *Aeromonas Hydrophila* Infection. Int J Agric Technol 2017;13(7.1): 1041-1064.
[31] Montoro P, Tuberoso CIG, Piacente S, Perrone A, De Feo V, Cabras P, Pizza C. Stability and antioxidant activity of polyphenols in extracts of *Myrtus communis* L. berries used for the preparation of myrtle liqueur. J Pharm Biomed 2006; 41:1614-1620.
[32] Mosha SS. A Review on Significance of Azolla Meal as a Protein Plant Source in Finfish Culture. J Aquac Res Development 2018;9: 7.
[33] Musa N, Ramly HR, Abdul Manaf MT, Razzak LA, Musa N. High water temperature impairs physiological responses in red hybrid tilapia: effects on cortisol and its regulation. AACL Bioflux 2017;10(5): 1297-1308.
[34] Naseem S, Bhat SU, Gani A, Bhat FA. Perspective on utilization of macrophytes as feed ingredient for fish in future aquaculture. Rev Aquacult 2020; 1 (19). DOI: 10.1111/raq.12475.
[35] Nwachi OF. An overview of the importance of probiotics in aquaculture. J Fish Aquat Sci 2013; 8 (1): 30-32.
[36] Olaniyi CO, Oladunjoye IO. Replacement value of duckweed (*Lemna minor*) in Nile tilapia (*Oreochromis niloticus*) diet. Transnatl J Sci Technol 2012; 2 (9): 54-62.
[37] Paglia, D.E. and Valentine, W.N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70: 158-169.
[38] Palacios E, Ibarra AM, Racotta IS. Tissue biochemical composition in relation to multiple spawning in wild and pond-reared *Penaeus vannamei* broodstock. Aquaculture 2000;185: 353-371.
[39] Prabina BJ, Kumar K. Dried Azolla as a nutritionally rich cost effective and immunomodulatory feed supplement for broilers. Asian J Anim Sci 2010;5: 20–22.
[40] Richter N, Siddhuraju P, Becker K. Evaluation of nutritional quality of Moringa (*Moringa oleifera* Lam.) leaves as an alternative protein source for Nile tilapia (*Oreochromis niloticus* L.). Aquaculture 2003;217 (1-4): 599-611.
[41] Robertson RP, Harmon JS. Pancreatic islet b-cell and oxidative stress: The importance of glutathione peroxidase, FEBS Letters 2007;581, 3743–3748.
[42] Shiomi N, Kitoh S. Culture of Azolla in a pond, nutrient composition, and use as fish feed. Soil Science and Plant Nutrition 2001; 47, 27–34. DOI:10.1080/00380768.2001.104 08365.
[43] Simčič T, Jesenšek D, Brancelj A. Effects of increased temperature on metabolic activity and oxidative stress in the first life stages of marble trout (*Salmo marmoratus*). Fish Physiol Biochem 2015; 41:1005-1014.
[44] Sithara K, Kamalaveni K. Formulation of low-cost feed using azolla as a protein supplement and its influence on feed utilization in fishes. Current Biotica 2008;2: 212–219.

ISSN- 2394-5125 Vol 8, Issue 1, 2021

[45] Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biol Med 1995;18, 321–336.
[46] Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J Agric Food Chem 1995; (43): 27-32.