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Effect of Kam Kung alga (*Chara corallina* Willdenow) on the growth performance and oxidative defense of Nile tilapia (*Oreochromis niloticus*)

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Abstract The result showed that the survival rate and FCR of the fish fed with algal inclusion diets at 7.5 and 10.0% were significantly differed ($p<0.05$) as compared to the control group. While, the fish fed with algal inclusion diets at 5.0, 7.5 and 10.0% were significantly differed in protein efficiency ratio of fish from control group ($p<0.05$). The percentage of protein content in fish carcass fed with experimental diets at 7.5 and 10.0% were significantly higher than the control group ($p<0.05$). All groups that received alga supplement did not show significant change in the level of malondialdehyde (MDA) in liver. At 2.5% inclusion found to be low lipid oxidation which was 96.39±13.08 nmol MDA/g liver. It was significantly differed ($p<0.05$) from the fish fed with the diets supplemented with algal powder at 5.0, 7.5 and 10.0 % which were 125.32±17.88, 122.24±18.50 and 122.52±25.60 nmol MDA/g liver, respectively. Glutathione and specific glutathione activity of fish fed with algal inclusion diets at 0 and 10.0% were significantly differed ($p<0.05$) as 91.34±30.42 and 164.26±41.50 nmol GSH/mL and 94.99±36.75 and 162.12±48.97 mmol GSH/mg protein, respectively. These findings indicated that the fish fed with 7.5% algal supplemented tended to optimal feed level for growth performance when evaluated at the 4th months and *C. corallina* led to increase the level of glutathione in liver of Nile tilapia.

Keywords: Edible algae, Freshwater, Lipid, Tilapia, Glutathione

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Introduction

Algae supplemented feeds has been practicing for a long time, in order to promote growth performance, health and coloring in aquatic animals, for example, *Haematococcus* for enhancing the flesh color in Trout (Sommer *et al*., 1992); *Spirulina* for promoting growth, fecundity, and immunity in fancy carp (Promya *et al*., 2012); *Cladophora glomerata* inclusion in Nile tilapia feed (Ruangsomboon and Choochote, 2004); and *Cladophora* spp. for *Pangasianodon* hybrid (Amornlerdpison *et al*., 2015) which enhanced growth efficiently. It has been reported of using *Spirulina* and *Cladophora* spp. supplemented feeds on African Sharptooth catfish in order to study growth, flesh quality and immunity. The results showed that 5% of *Spirulina platensis* inclusion simulated the immunity and enhanced the carotenoid in fish flesh (Promya and Chimanat, 2011). Using *S. platensis* as feed additive, increased antioxidant protective capacities in mono-sex Nile tilapia and hybrid red tilapia (*Oreochromis mossambicus* x *O.niloticus* (Amer, 2016; Ungsethaphand *et al*., 2010). The seaweed, *Ulva lactuca*, *Sargassum* sp. and *Taonia atomaria* could be supplemented to diets for Nile tilapia (Azaza *et al*., 2006; Pratiwy *et al*., 2018; Hussein, 2017).

Antioxidant can be classified into enzymatic antioxidants and nonenzymatic antioxidants such as vitamin C, β-carotene, glutathione peroxidases and catalase (Yangthong *et al*., 2009; Roy *et al*., 2021). Antioxidants are natural substances which may prevent or delay some types of cell damage (Jebur *et al*., 2016; Kumar, 2014). Antioxidant molecules produced by algae are used to protect the cell against reactive oxygen species (ROS) produced in response to biotic or abiotic stressors (Goiris *et al*., 2015). Oxidative stress had been assessed by determining the damage from free radicals by measuring the biomolecule which had altered or impaired by free radicals. The sensitive biomolecules to oxidation reaction by free radicals were lipids, protein and DNA. Lipids were sensitive to the reaction because it is the major composition in cell wall structure. When lipid was oxidized by free radicals, the end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Marcogliese *et al*., 2005; Rueda-Jasso *et al*., 2004).

Kam Kung alga (*Chara corallina* Willdenow) is the freshwater brittle wort (Charophyte) for human consumption (Chankaew *et al*., 2020). The alga had as great amount of nutrition as the other freshwater macroalgae, with high protein content, chlorophyll, carotenoids as well as the useful bioactive ingredients and antioxidants (Chankaew *et al*., 2020). According to Kam Kung alga, it has been reported that the alga has active ingredients which are beneficial for health, however, the study on utilization of this alga as aquatic animal food are unknown. Additionally, the low quality of this algae are found in dry season, which is not suitable for consumption. For value added, in this research the alga was used as a feed

ingredient for Nile tilapia, which is one of the most important cultured freshwater fishes in the world.

The research finding aimed to investigate the effect of the brittle wort, *C. corallina* as a dietary supplement on growth performance, feed utilization, body composition and to determine the oxidative stress prevention in Nile tilapia.

Materials and methods

Experimental design

The experiment was Completely Randomized Design (CRD). The experimental feeds were supplemented with the alga at 5 levels (treatments) of 0, 2.5, 5.0, 7.5 and 10.0% with 3 replicates and the experimental period was 120 days.

Feed preparation

All feed ingredients were weigh, then the dry stuffs were mixed in the mixture for 10 min, next, the oil was added, and mixed again for 10-15 min. After that 30-35% of water was added and mixed homogenously about 10-15 min. The dough was then put through the pelleting machine with 2.5 mm of pellet diameter and evenly cut. The pellets were then oven dried at 60 ℃ for 10-15 hr. After cooling down and sieving the dust, the feed was stored in plastic bags and kept refrigerated at 4 ℃. Nutrition compositions of experimental feeds were also analyzed.

Experimental feeds

The proximate compositions of Kam Kung alga was determined following AOAC (2016); it is presented in Table 1. The protein and lipid in experimental feeds were formulated at 30 and 6 %, respectively. The formulations of experimental feeds are showed in Table 2.

Table 1. Proximate compositions of *Chara corallina* in the experimental diets (dry weight basis)

Nutrition compositions	% of algal dry weight
Crude protein	16.87±0.11
Crude lipid	0.59±0.04
Crude fiber	19.17±0.52
Ash	15.56±0.00
Moisture	16.65±0.03
Carbohydrate	49.68±0.04

Table 2. Ingredients of the experimental diets (g/100 g feed)

Ingredients	**Feed formulations** (g/100 g feed)				
C. corallina meal	0	2.5	5.0	7.5	10.0
Fish meal	10	10	10	10	10
Poultry meal	12	12	12	12	12
Soybean meal	35.22	34.33	33.45	32.55	31.67
Rice bran	15	15	15	15	15
Broken rice	23.63	22.1	20.5	18.93	17.34
Oil	2.9	2.87	2.85	2.82	2.79
Salt	0.2	0.2	0.2	0.2	0.2
Vitamins and minerals	1	1	1	1	1
Total	100	100	100	100	100

Experimental fish and culture system

Nile tilapia from the private farm were transferred and nursed in fiber tanks. They were fed twice a day for 2 weeks. The experimental fish with average weight of 10.73 g were sampling into the 250 L fiber tanks with 175 L of water. The stocking number was 25tails/tank. They were fed with experimental feeds until satiation twice a day for 120 days. Aeration was supplied throughout the experimental period. Tank cleaning and 80% water exchange were done every other day.

Fish behavior, feeding behavior and other symptoms of fish during experimental period were observed. Growth and survival of experimental fish were measured and recorded every 15 days for 120 days. The growth performance, feed utilization and survival were estimated in terms of average weight gain, average daily growth (ADG), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate which were calculated as follows:

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

$$\text{Weight gain (g)} = \text{final average weight} - \text{initial average weight}$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{\ln(\text{final average weight}) - \ln(\text{initial average weight}) \times 100}{\text{days}}$$

$$\text{Average daily growth (ADG, g/day)} = \frac{\text{final weight} - \text{initial weight}}{\text{days}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{total feed weight}}{\text{fish weight gain}}$$

$$\text{Average weight (g)} = \frac{\text{total fish weight}}{\text{number of fish}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{fish weight gain}}{\text{protein intake}}$$

Oxidative defense of Nile tilapia

Malondialdehyde (MDA) measurement

MDA was determined to assess the antioxidant activity from lipid peroxidation (LPO) reaction. TBARS Assay Kit by Cayman Chemical was used, following modified method of Amornlerdpison *et al*. (2015). The liver sample (40 mg) with buffer solution was homogenized at 1,600 g, 4 ℃ for 10 min. The supernatant (100 µl) was collected and mixed with 100 µl sodium dodecyl sulfate (SDS), after that 4 ml of color reagent was added. The samples were boiled at 90 ℃ for 60 min, and then centrifuged at 1,600 g at 4 ℃ for 10 min. After that the sample was left under room temperature for 30 min. The clear solution, 150 µl, were collected and transferred into 96 well plate. The absorbance was read at 540 nm by microplate reader (Biochrom, 2000). The concentrations were calculated from the standard curve equation, as following formula.

Concentration of MDA for samples (nmole/ mL) = $(S_a / S_v) \times D$

Where, S_a = Amount of MDA in unknown sample (nmole) from standard curve, S_v = Sample volume (ml) added into the wells and D = Sample dilution factor.

Glutathione (GSH) measurement

Glutathione Assay Kit by Cayman Chemical (Sigma-Aldrich; MAK 085) was used which modified method of Amornlerdpison *et al*. (2015). The liver sample (30 mg) was washed with PBS buffer, then immediately immerse in dry ice, homogenized with 5 % Sulfosalicylic acid (SSA), incubated for 10 min at 2 - 4 ^{o}C and centrifuged at 10,000 x g, for 10 min at 4 ° C. Then, 10 µl of supernatant were collected and mixed with 150 µl cocktail solution, which contained with buffer, co-factor mixture, enzyme mixture, DTNB (dithiobis 2-nitrobenzoic acid). After that the sample was left under room temperature for 5 min then added 50 of NADPH solution. The absorbance, with 4 min of kinetic reaction, was read at 405 nm by microplate reader (Biochrom, 2000). The concentrations were calculated from the standard curve equation, as following formula. The total protein content in the supernatant was measured using the method of Lowry *et al*., (1951). Total glutathione (GSH) were calculated from the GSH standard curve equation, as following formula.

$$\text{nmoles GSH per mL of sample} = \frac{\Delta A_{405}/\text{min (sample)} \times \text{dil}}{\Delta A_{405}/\text{min(1 nmole)} \times \text{vol}}$$

Where, A405/min (sample) = slope generated by sample (after subtracting the values generated by the blank reaction), A405/min (1 nmole) = slope calculated from standard curve for 1 nmole of GSH, dil = dilution factor of original sample, and vol = volume of sample in the reaction in mL.

Statistical analysis

All data from measurements were verified for normality, and analyzed by variance analysis (One-way ANOVA). When the ANOVA identified differences among treatments, multiple comparisons among means were made with Duncan‘s new multiple range test. Mean differences between treatments were tested for significance ($p<0.05$). Values are expressed as mean ± standard deviation (sd) of means of three replications.

Results

Nutrition compositions of experimental feeds

Nutrition compositions of experimental feeds were shown in Table 3. The protein contents were closed to the designed levels (30%). There were no statistically significant differences of protein and lipid contents of experimental feeds ($p>0.05$).

Growth performances

Growth performance and feed efficiency of Nile tilapia fed with experimental diets for 120 days were shown in Table 4. Survival rate of Tilapia fed with 5.0%, 7.5% and 10.0% of algal supplemented feeds were statistically higher than those of the fish in control group and 2.5% supplemented feed ($p<0.05$). There were no statistical differences of average weight, weight gain, average daily growth and specific growth rate of all treatments ($p>0.05$). The highest survival rate was found in Nile tilapia fed with 10% algal supplemented feed at 97.33±2.31%. The fish fed with 7.5% algal supplemented feed had the lowest FCR of 1.31 ±0.06 and the highest PER of 2.22 ±0.11 which were statistically significant differences ($p<0.05$) with the FCR and PER of the control group. No significant differences were found of both FCR and PER among the fish fed 7.5% and 10.0% and 5.0% supplemented feed, and between the fish fed 2.5% supplemented feed and control group. However, the Nile tilapia fed supplemented feed at 5.0%, 7.5% and 10.0% had the better FCR and PER than that of the control group.

Table 3. Proximate composition of the experimental diets (g/100 g feed)

Chemical composition	Diets (level of *C. corallina* replacement)				
	1 (0%)	**2 (2.5%)**	**3 (5.0%)**	**4 (7.5%)**	**5 (10.0%)**
Crude protein	29.09±0.13	29.15±0.07	30.87±0.11	29.84±0.17	30.95±0.04
Crude lipid	8.22±0.40	8.02±1.89	9.41±0.30	8.42±0.78	9.51±0.33
Crude fiber	0.50±0.45^{b}	0.46±0.40^{b}	0.50±0.30^{b}	0.36±0.20^{b}	1.33±0.23^{a}
Ash	4.28±0.41^{c}	4.00±0.17^{c}	4.09±0.26^{c}	4.88±0.05^{b}	5.63±0.06^{a}
Moisture	3.08±0.27	3.67±0.46	3.16±0.63	3.64±0.42	3.49±0.19

The presented data were average mean±sd, the superscripts in the same column show statistical differences of means ($p<0.05$).

Table 4. Growth performance and feed utilization of the Nile tilapia fed *C. corallina* as feed additive in diets

Growth performance	Diets (level of *C. corallina* replacement)				
	1 (0%)	2 (2.5%)	3 (5.0%)	4 (7.5%)	5 (10.0%)
Survival rate (%)	62.67±8.23^{b}	77.33±4.62^{b}	96.00±0.00^{a}	96.00±4.00^{a}	97.33±2.31^{a}
Average weight (g)	90.04±6.75	95.04±16.84	88.43±5.52	91.75±1.07	84.27±6.37
Average weight gain (g)	70.27±6.93	79.95±10.52	72.96±3.89	77.03±3.46	68.20±5.05
Average daily growth (g/day)	0.78±0.08	0.89±0.11	0.81±0.04	0.85±0.04	0.76±0.06
Specific growth rate (%)	2.24±0.10	2.37±0.12	2.28±0.05	2.33±0.04	2.22±0.07
FCR	1.77±0.17^{a}	1.71±0.33^{ab}	1.40±0.07^{bc}	1.31±0.06^{c}	1.38±0.13^{bc}
PER	1.48±0.24^{c}	1.67±0.40^{bc}	2.05±0.11^{ab}	2.22±0.11^{a}	2.10±0.22^{ab}

The presented data were average mean±sd, the superscripts in the same column show statistical differences of means ($p<0.05$).

Carcass composition of Nile tilapia

Results of diet which effected on Nile tilapia body chemical composition at the end of the feeding trial are summarized in Table 5. A slight difference between the five treatments was observed. It was found that in Nile tilapia carcass of the fish fed algal supplemented feeds at 7.5% and 10.0%, had statistically significant higher protein content ($p<0.05$) than the control group. The highest protein levels were 58.04±0.77 % and

57.93±2.64 %, respectively. The fiber content of the carcass of fish fed 10.0% algal supplemented feed was 0.33±0.13 % and statistically higher than that of the control group ($p<0.05$). Whereas, the highest ash was 24.59±2.73, which found in carcass of fish fed with 5.0% algal inclusion feed. It was also statistically higher than that of the control group ($p<0.05$). Nile tilapia fed with the algal supplemented feed at all levels had no significant differences of lipid content ($p>0.05$).

Table 5. Carcass composition (% dry weight) of Nile tilapia after 120 days

Carcass composition	**Diets (level of *C. corallina* replacement)**				
	1 (0%)	**2 (2.5%)**	**3 (5.0%)**	**4 (7.5%)**	**5 (10.0%)**
Crude protein (%)	52.12±0.13^{b}	54.70±0.18^{ab}	55.15±1.58^{ab}	58.04±0.77^{a}	57.93±2.64^{a}
Crude lipid (%)	10.05±1.75	8.03±2.50	4.71±4.16	4.31±3.35	4.50±2.76
Crude fiber (%)	0.02±0.04^{b}	0.18±0.27^{ab}	0.24±0.07^{ab}	0.16±0.04^{ab}	0.33±0.13^{a}
Ash (%)	19.74±1.00^{b}	21.71±0.94^{b}	22.71±1.85^{ab}	24.59±2.73^{a}	22.13±2.24^{b}
Moisture (%)	72.82±1.98^{b}	75.30±1.32^{ab}	75.23±2.14^{ab}	76.60±0.59^{a}	77.25±1.02^{a}

The presented data were average mean±sd, the superscripts in the same column show statistical differences of means ($p<0.05$).

Oxidative defense of Nile tilapia

Oxidative stresses in Nile tilapia fed with experimental feeds were assessed at 4th month of culture. The liver of fish were sampling for malondialdehyde (MDA) and Glutathione (GSH) measurement, respectively.

Malondialdehyde (MDA)

The levels of algal inclusion had affected on the lipid oxidation (LPO) but there was no significantly different from the control group. The Nile tilapia fed with the lowest algal inclusion feed at 2.5%, had lower LPO than at 5.0,7.5 and 10.0 % was 96.39±13.08 nmol MDA/g liver and was statistically different of LPO ($p<0.05$), 125.32±17.88, 122.24±18.50 and 122.52±25.60 nmol MDA / g liver, from fish fed algal supplemented feeds at 5.0%, 7.5% and 10.0%, respectively (Figure 1).

Glutathione (GSH)

GSH in fish liver had elevated as the algal inclusion levels increased. Nile tilapia from the control group, had low of GSH as 91.34±30.42 nmol GSH/mL sample, with no significant difference ($p>0.05$) with the GSH in fish fed with 2.5 and 5.0% of algal supplemented feeds, but had statistically lower than the fish fed algal supplemented feeds at 7.5 and 10.0% ($p<0.05$). The specific glutathione activity was found in correlate

with GSH presented. The fish fed the highest algal inclusion had higher GSH than the fish fed low algal inclusion at 2.5 and 5.0% ($p<0.05$) (Table 6).

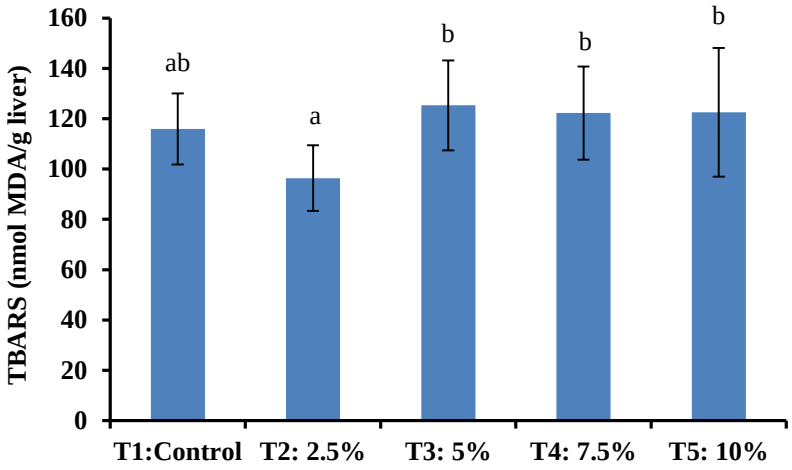

Figure 1. Lipid peroxidation (LPO) of Nile tilapia fed with experimental feeds supplemented with different levels of *C. corallina* for 120 days. Bars represent ± standard deviation (n=6), columns annotated with the same letters are not statistically different ($p>0.05$)

Table 6. Glutathione (GSH) of Tilapia liver fed with experimental diets supplemented with different levels of *C. corallina* for 120 days

Diets (level of *C. corallina* replacement)	**glutathione** (nmol GSH/mL sample)	**Specific glutathione activity** (mmol GSH/mg protein)
1. control	91.34 ± 30.42^{a}	94.99 ± 36.75^{a}
2. (2.5%)	111.26 ± 25.70^{ab}	104.99 ± 13.01^{a}
3. (5.0%)	113.14 ± 20.74^{ab}	90.32 ± 11.34^{a}
4. (7.5%)	140.97 ± 23.73^{bc}	124.87 ± 31.79^{ab}
5. (10.0%)	164.26 ± 41.50^{c}	162.12 ± 48.97^{b}

The presented data were average mean±sd, the superscripts in the same column showed statistical differences of means ($p<0.05$).

Discussion

Growth performances and nutrition compositions of Nile tilapia carcass

The Nile tilapia fed with Kam Kung alga supplemented feeds at 0, 2.5, 5.0, 7.5 and 10.0% for 120 days, it could be concluded that at 7.5%, growth performances of the fish were the best results, with low FCR and

high survival rate and protein efficiency ratio (PER), and had statistically significant differences with the control group. The similar research is reported by Ruangsomboon and Choochote (2004), study the effects the diets supplemented with fed with green alga, *Cladophora glomerata* on growth performance of Nile tilapia as added to basal diet at 0, 2.5,5.0 and 7.5 %, the FCR of feeding diet containing was 2.5-7.5 % was not significantly different from the control. Using the *C. corallina* for Nile tilapia diet had lower FCR than *C. glomerata*, while the optimum level of feed supplement was the same level (7.5%) (Ruangsomboon and Choochote, 2004). Due to the cellular structure of *C.corallina* which contained cellulose (Peerapornpisal, 2006), and hard to digest, resulted in the decreasing growth in the fish fed the highest level supplement (10%). In contrast, increasing of *Gracilaria fisheri* in fish feed was not effected on to the growth Nile tilapia (Phromkerd, 2014). Normally, high dietary fiber levels could reduce food utilization and digestibility (Anderson *et al*., 1984).

The body composition of protein were significant differed from the control treatment ($p<0.05$). It is possible that if the higher amount of alga is added into the feeds, the carcass protein content would be higher. In contrast, the study of green seaweed, *Caulerpa racemosa* supplemented feeds at 2.5, 5.0, 7.5, 10.0 and 12.5% on growth and nutrition compositions of Red tilapia (*Oreochromis* sp.) for 120 days, the results showed that fish fed the supplemented feeds yielded the lower protein and lipid contents than the control group (Wattanakul *et al*., 2016). While, diet 5% supplement of *Sargassum* spp. could be improved the carcass quality without any adversely affected on sex-reversed tilapia (*Oreochromis niloticus*) growth performance (Yangthong *et al*., 2014).

Oxidative defense of Nile Tilapia

Malondialdehyde (MDA)

The occurrence of oxidative stress in fish is caused by age, nutrient and nutrition, toxins, disease and xenobiotic. Nutrients and nutrition could be stimulated or reduced oxidative stress in fish (Yangthong, 2014). Ratanapot *et al*. (2002) concluded that the inclusion of green alga, *Spirogyra* sp. in fed for Nile tilapia at the 5-10% for 4 months, it could statistically reduce the LPO in liver and kidney. The green alga, *Cladophora* spp. inclusion at 5-10% had statistically reduced the lipid peroxidation in hybrid catfish, *Pangasianodon* (*P.gigas* × *P. hypophthalmus*), in the 5^{th} and 7^{th} month of culture (Amornlerdpison *et al*., 2015). Adding two green algae to Nile tilapia as feed could improve fish health because antioxidants from the algae would boost fish immunity and tolerance to abusive environments and diseases, resulted in high survival rate**.** In this study, Nile tilapia fed with 2.5% of algal supplemented feed tended to reduce the LPO. Whereas, the fish fed with higher percentage of inclusion of 5.0, 7.5 and 10.0%

showed high levels of MDA and there was not significantly differed when compared to the control. Therefore, further experiments might be done with the tendency of MDA parameters on stressed fish and challenge with the pathogen in Nile Tilapia (Yangthong *et al*., 2012).

Glutathione (GSH)

C. corallina as feed supplement increased GSH in liver sample of Nile tilapia when fed the feeds for 4 months. The fish fed high algal inclusion at 10.0 %, showed higher GSH than the control group and lower inclusion levels. In contrast, the report in hybrid catfish, *Pangasianodon* (*P. gigas* × *P. hypophthalmus*) fed with *Cladophora* spp. inclusion feeds at 2.5-10.0% had not affected on the GSH in red blood cells (Amornlerdpison *et al*., 2015). It might be due to the different organs which used to determine the GSH. Rodriguez-Ariza *et al*. (1994) reported that liver tissue responded to GSH in fish. Glutathione is the antioxidant which helps to eliminate free radicals from the body, increasing antioxidant activity (Doyotte *et al*., 1997), protecting liver cells and alleviating neuron intoxication (Raghunathan *et al*., 2007). In this study, the fish fed with *C. corallina* supplemented feed at 2.5% tended to reduce LPO and when the fish fed the feeds with algal inclusion at 7.5-10.0%, the GSH in liver increased.

In conclusion, *C. corallina* alga meal could be used as a feed supplement for Nile tilapia at dietary inclusion level of 7.5%, could gain optimal growth performances, the highest carcass protein content and the lowest lipid retention. All groups received alga supplement were not significantly decreased the level of MDA in liver of Nile tilapia but increased of glutathione. The *C. corallina* showed a potential to be supplemented as feed ingredient in aquaculture with the value added of this alga. Further studies would be focused on digestibility coefficients of this alga.

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