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The Duration of Protection Conferred by Garlic on African Catfish (*Clarias gariepinus*) Against *Aeromonas hydrophila*

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Abstract

This study aims to investigate the efficacy of dietary doses of garlic (*Allium sativum*) peels and cloves in growth performance, disease resistance and the duration of protection to African catfish (*Clarias gariepinus*) juvenile against *Aeromonas hydrophila* infection. African catfish juvenile was fed twice daily for 4 weeks with commercial catfish diet (Control) and 20 gkg^{-1} of garlic peels and cloves which incorporated into the fish formulated diet. After 4 weeks of post feeding, 15 fish were randomly selected for challenge test with 10^8 cell/mL of *A. hydrophila* and fed control diet. The duration of the protection was observed at 7, 14 and 21 days following infection. The result demonstrated that the duration of protection of garlic toward African catfish against *A. hydrophila* at 7, 14 and 21 days after stopped feeding with garlic inclusion diet provided protection until 14 days and slightly reduced protection after 21 days. However, the survivals of treatment groups were still higher compared to the control group. The results indicated that garlic cloves showed better performance in enhancing the African catfish disease resistant towards infection by *A. hydrophila*.

Keywords: Garlic clove; Garlic peels; Phytobiotic; African catfish; *Aeromonas hydrophila*

Introduction

Plant products application in fish culture have become famous for increasing the defence system activity as well as disease protector against various pathogens such as bacteria and fungi. Garlic is one of the traditional herbs that recently used as antimicrobial in fish disease [1]. Its active substances which are allicin and flavonoids were proven to have strong antimicrobial properties. The immunostimulants usages possibly activate the organism antibody complement pathway thus providing resistant to the infection. The use of garlic as immunostimulant in diet has been pointed out in improving African catfish disease resistance against *A. hydrophila* infection. Its active components were proven to have strong antimicrobial properties since significantly reduced mortality of African catfish were obtained in research [2]. However, the duration of protection conferred by garlic is not well known. Research by Nya [3] stated that, the long-term duration of immunostimulants application leads to immune suppression. Therefore, the duration of protection by garlic on African catfish challenged with *A. hydrophila* was studied in order to observe the effect towards African catfish after fed with garlic (peels and cloves) at 20 gkg^{-1} of inclusion level. The ability of garlic to maintain in African catfish and provide protection against *A. hydrophila* was observed. Furthermore, minimal application of inclusion diet as antimicrobial in fish disease could be suggested.

Materials and Methods

Experimental fish

African catfish juvenile with average length of 14 ± 2 cm and weight of 16 ± 2 g was obtained from private farm in Pulau Bintongan, Rembau, Negeri Sembilan, Malaysia. The fish were transported in polythene bag, which supported with aeration. Later, fish were carefully transferred into two polythene tank of size 107 cm × 73 cm × 30 cm and left undisturbed for overnight. The fish were acclimatized for a week under aerated conditions at 28 ± 1.5°C. Fish were fed with a commercial catfish feed (Keli Cargill) at *ad libitium* twice daily during the acclimatization period.

Experimental diet

The garlic peels were separated from the cloves using a household knife. The peels and cloves were washed, and oven dried at 55°C for 15 min then grinded into fine powder using heavy duty blender (Panasonic). The powders (peels and cloves) were further incorporated into basal diet at inclusion level of 20 gkg^{-1}. No inclusion of either peels or cloves (0 g) was treated as a control. A total of 300 ml water was added to the dry ingredients and mixed thoroughly using mixer (Artisan – Kitchen mixer D300T) for 20 min at low speed until it became dough. The dough was further made into pellet using pellet machine (TJS22 Dual- Purpose machine) and dried using oven at 60°C for 24 h. The modified feeds were stored in screw cap bottle at room temperature for further use.

Experimental design and feeding trial

African catfish juvenile (n=225) were randomly divided into three groups (T1, T2, and T3) in the aquarium (0.6m × 0.3 m × 0.3 m, water volume 40 L) after acclimatization period. Each group consisted of 25 fingerlings with three replicates. Each aquarium was supplied with continuous aeration. Group T1 was fed with basal diet without inclusion of either garlic peels or cloves and treated as control (0 g). The remaining groups were fed with experimental diet with inclusion of garlic cloves (T2) and peels (T3) at 20 gkg^{-1} respectively. The fish were fed twice daily at 08:00 and 17:00 h *ad libitum* for four weeks.

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Growth performance

Growth performance and feed utilization including weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER) and feed conversion ratio (FCR) were determined for both control and experimental groups following these formulas:

- WG=Final weight (g) – Initial weight (g)
- SGR=100 × [(Ln of final weight) – (Ln of initial weight)]/ Experimental days
- FCR=Feed consumed (g)/Weight gain (g)
- PER=Wet weight gain (g)/Total protein intake (g)

Bacterial pathogen preparation

Aeromonas hydrophila was cultured in Trytic Soy Broth (TSB) (Soybean- Casein Digest Medium) Bacto, USP and EP specifications for 24 h at 30°C. The broth culture was centrifuged at 5000 × g, 27°C for 10 min. The supernatants were discarded, and the pellets were re-suspended in saline water (0.9% w/v). The optical density (OD) of the supernatants were examined at 550 nm of absorbance using spectrophotometer (Eppendorf Biophotometer Plus, Brand Tech). The bacterial suspensions were further diluted using standard dilution technique with saline water.

Experimental challenge

At the end of feeding trial experiment (4 weeks), 15 fish from each treatment group were randomly selected and divided into three groups. Each fish was injected with 0.1 mL of *A. hydrophila* by Intraperitoneal injection (IP) at concentration of 10^8 cellmL^{-1} at three different duration time which were; day seven post – feeding trial (G1), day 14 post-feeding trial (G2) and day 21 post-feeding trial (G3) (Figure 1). Mortality and survival percentage of fish in each duration group were recorded and calculated after 50% mortality of treatment that had no inclusion of garlic peels and clove (control group) achieved.

Evaluation of water quality parameter

Water exchange (50%) was done twice a week while the water quality was monitored daily. The pH, dissolved oxygen concentration and temperature were maintained at 6.5 ± 0.3, 5.0 ± 0.5 ppm and 27 ± 0.2°C, respectively, throughout the experiment. The pH was measured using pH meter (Jenway 3305) while the temperature and dissolved oxygen were determined by DO meter (YSI-model57).

Histopathological determination

Internal organs were collected from the fish before and after challenged with *A. hydrophila* for histopathology determination. Liver and spleen were taken out from each group. The samples were fixed in alcohol solution for 12 h and embedded in paraffin following routine processing. Then, the samples were dehydration and infiltration on using automatic tissue processor (Leica ASP 300S, Germany). The tissues samples were embedded in paraffin and were sectioned using rotary microtome (4 to 5 µm) (Leica, Germany) and stained with hematoxylin and Eosin (H&E) (Carleton et al., 1967). After that, the slides were examined under advance digital microscope (Leica E75).

Statistical analysis

Data for growth performance and feed utilization efficiency (WG, SGR, PER and FCR) were analysed using one-way Analysis of Variance (ANOVA). Hemotological values were analysed using t-paired test while survival percentage at each duration time were analysed using two-way Analysis of Variance (ANOVA) and significant differences ($p<0.05$) of means among treatment were compared using Turkey's multiple range test using SPSS version 20.

Results

Growth performance

The growth performance parameters of African catfish in terms of WG, FCR, SGR and PER of fish fed with 20 gkg^{-1} inclusion level of garlic (peels and cloves) for 4 weeks were presented in Tables 1 and 2. Garlic peels and cloves at 20 gkg^{-1} inclusion level had no significant ($p>0.05$) impact on WG, SGR, FCR and PER of African catfish fingerling as compared to the control group (Tables 1 and 2).

Survival of African catfish against *Aeromonas hydrophila*

The results of survival rate of African catfish after challenged at three different duration 7th, 14th, and 21st days post – feeding trial (4 weeks) with different groups of fish dietary (garlic peels and cloves) showed that, the garlic inclusion affected the survival rate of African catfish significantly ($p<0.05$) (Figure 2). The higher survival rate was observed in fish that fed with 20 gkg^{-1} garlic cloves (T2) followed by 20 gkg^{-1} of garlic peels inclusion group (T3). The protection of garlic slightly decreased at days 21 post – feeding trial. However, garlic treatment showed the highest survival as compared to the control group. The results from Tukey's multiple comparison also showed groups 14 days post feeding showed significant difference ($p<0.05$) compared with groups 7 and 21 days post feeding.

Histopathology of African catfish internal organ after infected with *Aeromonas hydrophila*

Histophatologic finding of the disease was observed in liver and spleen tissue of African catfish. The summary of histophatology distribution of liver and spleen at different duration was completely defined in Table 3. The liver section of African catfish fed on control diet (T1) showed the sign of necrosis, haemorrhage, lymphocytes infiltration and white blood cell in sinusoids (Figures 3a and 3b). All signs were observed at all duration time of day 7, 14 and 21 post-feeding trial. However, the sign of lymphocytes infiltration was observed only at day 7 and 14 post – feeding. Liver section of African catfish fed with 20g kg^{-1} garlic cloves (T2) and 20 gkg^{-1} garlic peels (T3) showed have no necrosis, lymphocytes infiltration and haemorrhage sign at all duration time (Figures 3c and 3d). White blood cells were also seen to be filled in sinusoids of African catfish liver section that treated with garlic (peels and cloves) (Figures 3c and 3d).

The spleen section of African catfish of control group (T1) showed the sign of necrosis, haemorrhage, and melanomacrophage (Figure 4a). All signs were observed at all duration time which were day 7, 14 and 21 post -feeding trial. However, the sign of melanomacrophage also observed at spleen section of African catfish fed with garlic (peels and cloves) treatments (Figures 4b and 4c). Moreover, spleen section of African catfish fed with 20 gkg^{-1} garlic cloves (T2) and 20 gkg^{-1} garlic peels (T3) showed only mild necrosis, and haemorrhage sign compared

Serial No.	Treatments
T1 (Control)	No inclusion of garlic peels and cloves
T2 (GC 20 g)	Inclusion of garlic cloves at 20 gkg^{-1}
T3 (GP 20 g)	Inclusion of garlic peels at 20 gkg^{-1}

Table 1: Experimental groups of African catfish fed with different dietary against *Aeromonas hydrophila* infection.

Treatment	Initial Weight (g)	Final weight (g)	Weight Gain (g)	FCR (g)	SGR (day/%)	PER (g)
T1 (Control)	406.67 ± 6.66	510.67 ± 13.54	104.00 ± 18.76	4.29 ± 1.38	3.25 ± 0.56	2.89 ± 0.52
T2 (GC 20 g)	406.67 ± 6.66	514.67 ± 18.41	108.00 ± 13.69	3.99 ± 1.64	3.35 ± 1.20	3.00 ± 1.13
T3 (GP 20 g)	400.00 ± 5.36	518.67 ± 19.33	114.67 ± 19.33	3.48 ± 1.85	3.58 ± 0.54	3.19 ± 0.93

Control: No inclusion of either garlic peels or cloves; T2: Inclusion of garlic cloves at 20 gkg^{-1} basal diet and T3: Inclusion of garlic peels at 20 gkg^{-1} basal diet, respectively; GC=Garlic Cloves; GP=Garlic Peels; FCR=Feed Conversion Ratio; SGR=Specific Growth Rate; PER=Protein Efficiency Ratio (Mean ± SE).

Table 2: Growth performance and feed utilization efficiency of African catfish fingerlings fed with different dietary inclusion of garlic.

Days	Treatment	Liver				Spleen			
		Necrosis	Haemorhage	White blood cell in Sinusoid	Lymphocyte infiltration	Necrosis	Haemorrhage	Melanomacrophage	Red and white pulp
(G1) 7th	T1 (Control)	++	++	++	++	++	++	++	-
	T2 (GC 20 g)	-	-	+	-	+	-	+	+
	T3 (GP 20 g)	-	-	+	-	+	-	+	+
(G2)14th	T1 (Control)	++	++	++	++	++	++	++	-
	T2 (GC 20 g)	-	-	+	-	-	-	+	++
	T3 (GP 20 g)	-	-	+	-	-	-	+	++
(G3) 21st	T1 (Control)	++	++	++	-	+++	++	++	-
	T2 (GC 20 g)	-	-	+	-	-	-	+	-
	T3 (GP 20 g)	-	-	+	-	-	-	+	-

Control (T1): No inclusion of either garlic peels or cloves; T3 Inclusion of garlic cloves at 20 g/kg basal diet, T6 Inclusion of garlic peels at 20 g/kg basal diet. Duration level: G1: Days 7 post – feeding trial, G2: Days 14 post – feeding trial, G3: Days 21post – feeding trial. Data were shown (-) none, (+) Mild, (++) Moderate, (+++) Severe.

Table 3: Summary distribution of liver and spleen histopathological findings of African catfish against *Aeromonas hydrophila* at different duration after stop feeding with experimental diet.

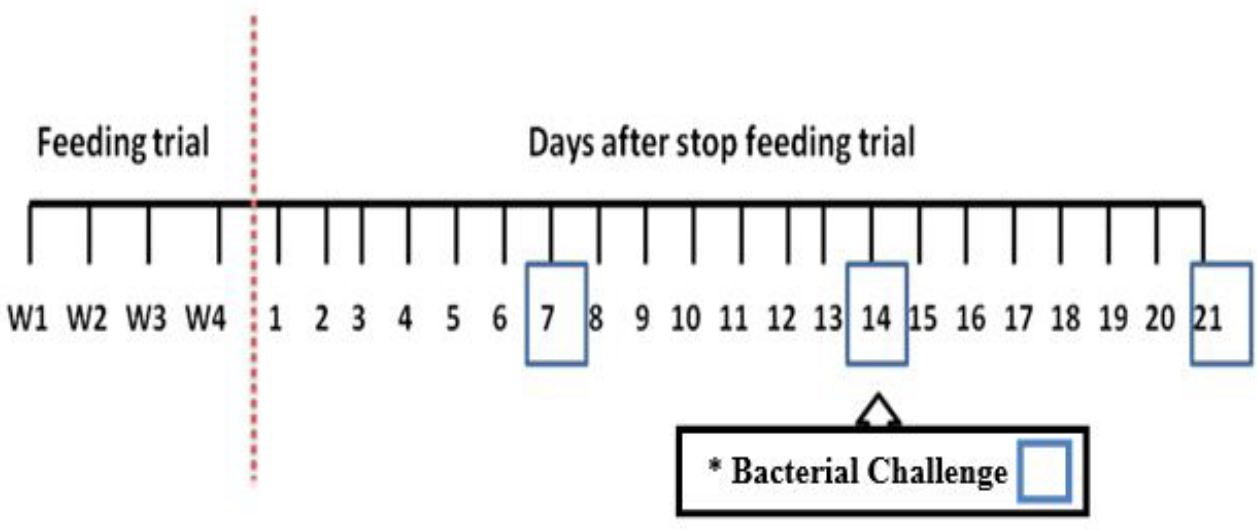

Figure 1: *In vivo* challenge assay time line of African catfish against *Aeromonas hydrophila* at different duration time after stop feeding trial.

with the control. Moreover, the spleen section appears of white and red pulp. However, white and red pulps are not well demarcated in most histologic sections of fish splenic tissue. Interestingly, basophilic sign was observed in spleen of African catfish fed with 20 gkg^{-1} garlic cloves (T2) only at days 7 post – feeding (Figure 4d). Nevertheless, this basophilic sign was not demonstrating in African catfish spleen fed with no inclusion of garlic (control) at all duration time.

Discussion

Garlic is one of the traditional herbs that have potential in disease resistance of African catfish. However, it did not affect the growth performance of the fish. According to the results, there were no significant differences ($p>0.05$) on growth performance in term of weight gain, FCR, SGR and PER of African catfish fed with 20 gkg^{-1} garlic (peels and cloves) for 4 weeks as compared to the control group

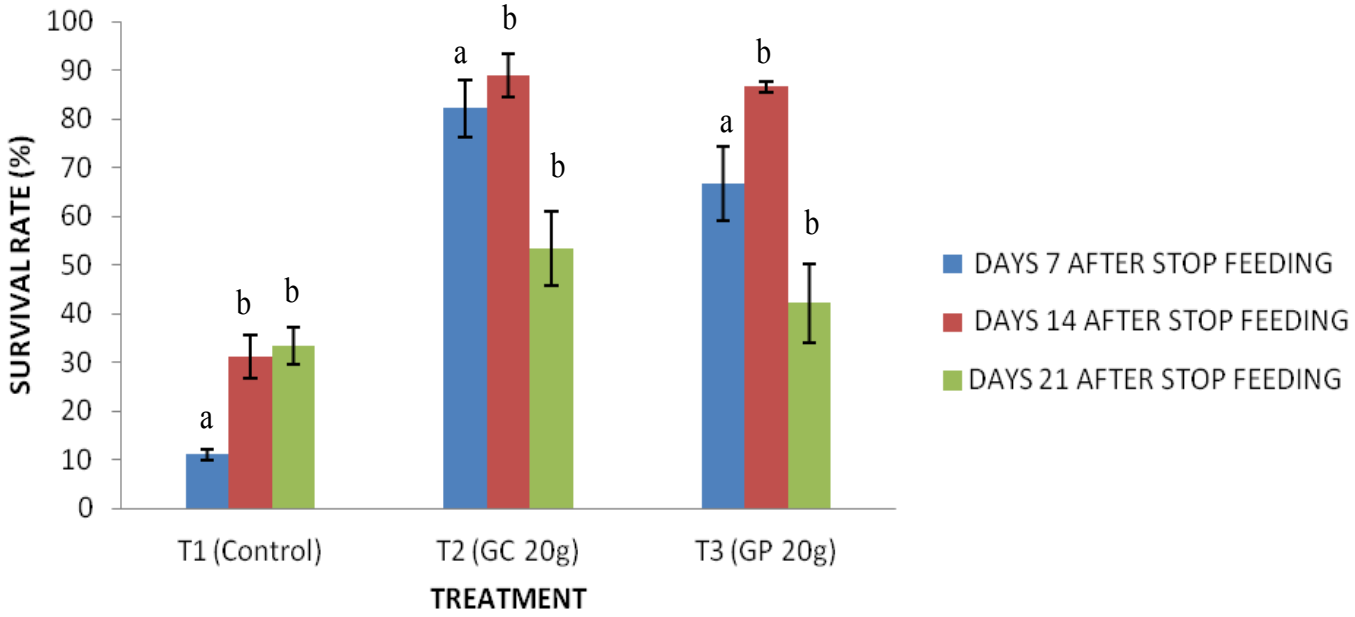

Figure 2: The survival rate of African catfish against *Aeromonas hydrophila* infection at different duration time.

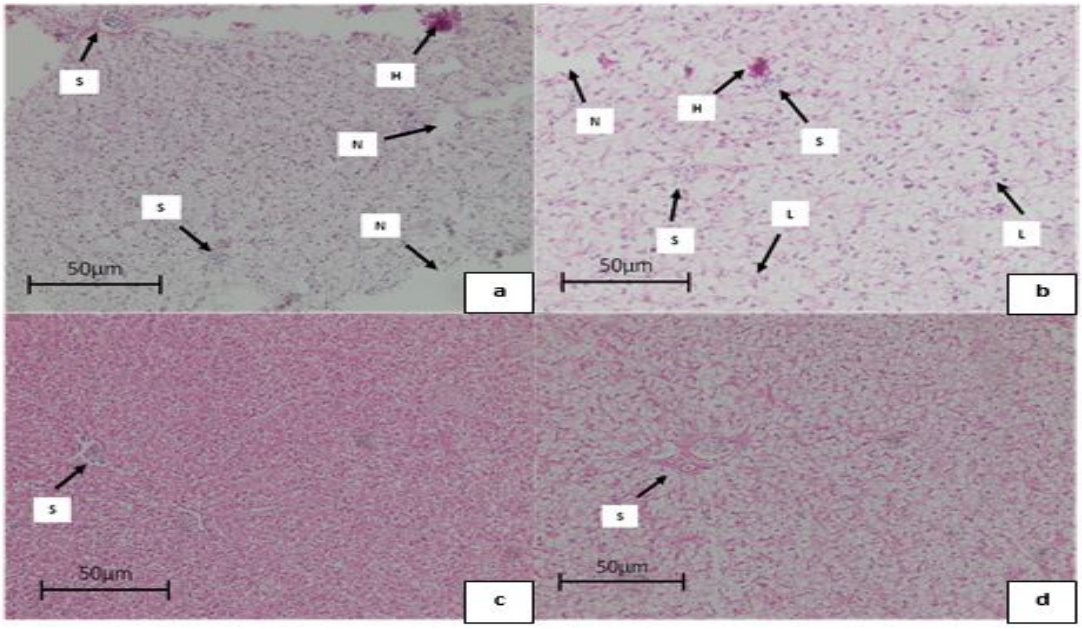

Figure 3: (a and b) Histopathology section of liver of African catfish that fed with no inclusion of garlic (peels and cloves) (Control, T1). The picture shows the sign of necrosis (N), haemorrhage (H), white blood cell in sinusoids (S) and lymphocyte infiltration (L) (40x). **(c)** Histopathology section of liver of African catfish that fed with inclusion of garlic cloves (GC 20 g, T2): The picture shows the sign of white blood cell in sinusoids (S) (40x). **(d)** Histopathology section of liver of African catfish that fed with inclusion of garlic peels (GP 20 g, T3): The picture shows the sign of white blood cell in sinusoids (S) (40x).

(T1). However, during feeding trial period, the formulated diet was consumed well by the African catfish and the behaviour of fish during the feeding trial was normal.

The results showed the sign of haemorrhage, necrosis and lymphocyte infiltration observed in liver of fish that fed with dietary which had no inclusion of garlic peels and cloves (T1). Meanwhile, no sign of haemorrhage and necrosis observed in liver of fish fed with dietary of garlic (peels and cloves) at inclusion level of 20 gkg^{-1} (T2 and T3). Previous study that had been done by Huizinga et al. [4] showed that necrosis sign was presence in liver and kidneys tissues of fish that infected with *A. hydrophila*. Necrosis of the liver was reported to be associated with toxins and extracellular products such as hemolysin and protease that produced by *A. hydrophila* [5]. Moreover, the haemorrhage

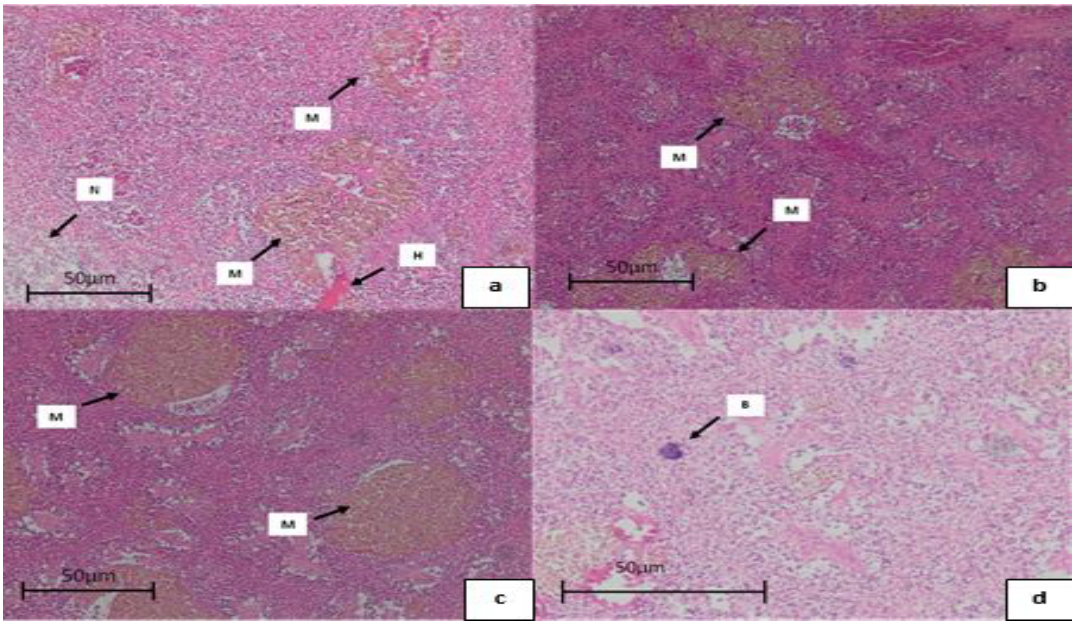

Figure 4: (a) Histopathology section of spleen of African catfish that fed with no inclusion of garlic (peels and cloves) (Control, T1). The picture shows the sign of necrosis (N), haemorrhage (H) and melanomacrophage (M) (40x). **(b)** Histopathology section of spleen of African catfish that fed with inclusion of garlic cloves (GC 20 g, T3). The picture shows the sign of melanomacrophage (M), red and white pulp (40x). **(c)** Histopathology section of spleen of African catfish that fed with inclusion of garlic peels (GP 20 g, T6). The picture shows the sign of melanomacrophage (M), red and white pulp (40x). **(d)** Histopathology section of spleen of African catfish that fed with inclusion of garlic cloves (GC 20 g, T3). The picture showing some formation and aggregation of the basophilic leukocytes (B) at day 7 after stop feeding trial. This is the plasma cells and its clonal expansions (40x).

and lymphocyte infiltration in liver showed a visceral haemorrhagic septicaemia. Indeed, the garlic (peels and cloves) inclusion helped to protect the fish from pathological damaged and changes in liver after infected with the pathogen. Observation of melanamacrophage sign in all spleen tissues section of fish in control and garlic treatments showed that the phagocytic activity occured in the host in order to fight with the pathogen. The melanomacrophage was found at the center of spleen infected with bacteria [5].

Moreover, the results of the white and red pulp observed in fish spleen fed with garlic inclusion could be the process of creating new blood cells in the body (hematopoises). Hematopoises usually occur within both spleen and kidney of the organism [6]. Garlic inclusion diet provided protection to African catfish until days 14 after stop feeding. The protection was slightly reduced at day 21 post – feeding. It could be due to immunosuppression where the long term of application of immunostimulants leads to reduction of the activation or efficacy of the immune system [7]. It was an agreement with Bagni M and Bricknell I [8,9] mentioned that long term exposure of plant herbs as immunostimulant in seabass lead to losing the compound sensitivity thereby reduce their protection. However, Kaleeswaran et al. [10] found that herbal diet reduce mortality *of Catla catla* infected with *A. hydrophila* even though at 21 and 28 days of feeding trial. This present study was agreement with [3] that showed the protection of garlic at rainbow trout reduced at days 28 received dietary garlic treatment. Therefore, it suggested the garlic inclusion should be added after days 14 to increase the efficacy as an immunostimulant. However, this current study showed that lower mortality observed among fish in garlic treatments as compared to the fish in control. The higher survival rate was observed in fish that fed with 20 gkg^{-1} garlic cloves (T2) followed by 20 gkg^{-1} of garlic peels inclusion group (T3) as compared to the control. This confirmed that active compound in garlic (peels and cloves) had strong antimicrobial properties.

Previous research [11] suggested that garlic functions are mainly attributed by the bioactive components including sulphur compounds such as allin, diallysulphides and allicin. Allicin is the main thiosulfinates which is sulphur compounds that contributed in healthy effects and act as immune-stimulation. In contrast Shobana et al. [12], mentioned that garlic peels containing of phenolics and flavonoid as active compound to prevent oxidative stress related disease. Moreover, Srinivasan [13] stated that flavonoid compound in outer layer of garlic are potent antioxidants and reportedly have a wide range of biochemical functions involving of immune system, gene expression, blood flow, and liver function. Therefore, these two active compounds in garlic peels and cloves promoted in enhancing the survival rate and protection of catfish at the different duration.

Conclusion

Thus, it can be concluded that the garlic has antimicrobial properties that able to enhance the organism productivity whenever it acts as appetite stimulation and improve the defense mechanism of African catfish.

Acknowledgments

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