Acute Toxicity of Garlic (*Allium sativum*) Extract to Snubnose Pompano (*Trachinotus blochii*) Juvenile

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Abstract

Garlic (*Allium sativum*) is a well-known medicinal herb which has been shown to possess anti-microbial and anti-parasitic properties. This study was conducted to test the toxicity levels of snubnose pompano (*Trachinotus blochii*) juvenile to garlic (*Allium sativum*) extract by determining the cumulative mortality and median lethal concentration (LC50). Test fish were exposed to six concentrations of the extract (2.5, 5.0, 7.5, 10, 20 and 30 ppm) in a 96-hour static bioassay. Cumulative mortality was highest at 100 % for 30 ppm garlic extract, with mortalities found to increase with increasing concentration. Test fish exposed to 20 and 30 ppm exhibited weak and static behavior. The LC50 of garlic extract to *T. blochii* was found to be 7.48 ppm at 96 h. Findings of the present study suggest that aqueous garlic extract up to 5 ppm can be safely used in pompano for prophylactic purposes.

Keywords: Median lethal concentration (LC50), garlic extract, pompano

Introduction

Aquaculture has emerged as the fastest growing food-producing sector in the past few decades (FAO, 2014) but has been affected with the emergence of parasitic diseases (Palma *et al*., 2015; Akoll *et al*., 2012; Nowak, 2007). Numerous anti-parasitic and chemical treatments have been used to control fish parasites. Although these existing therapeutants are effective against ectoparasites, herbal or natural extracts are an environment-friendly alternative. There have been numerous reviews about the potential use of medicinal herbal extracts in aquaculture (Bulfon *et al*., 2013; Reverter *et al*., 2014; Van Hai, 2015). Moreover, the effectiveness of plant-derived substances in controlling fish parasites have been widely studied (Picon-Camacho *et al*., 2012; Erguig *et al*., 2015; Wunderlich *et al*., 2017; Tavares-Dias, 2018). Garlic (*Allium sativum*) is one of the herbal remedies that help in the control of fish pathogens. Allicin has been identified as the major active pharmaceutical molecule found in crushed garlic. It is produced when the chemical compound alliin is catalyzed by the enzyme allinase which happens when the garlic bulb is crushed, sliced or processed mechanically (Tracy and Kingston, 2007).

It has been reported that garlic extract has antihelminthic properties against *Capillaria* sp. in the common carp (*Cyprinus carpio*) (Peña *et al*., 1988), inhibitory effects for fish parasite infestation against *Ichthyopthirius multifiliis* (Buchmann *et al*., 2003) in Nile tilapia (*Oreochromis niloticus*), trichodiniasis in eel (*Anguilla anguilla*) (Madsen *et al*., 2000), *Neobenedenia* sp. (Militz *et al*., 2013) in farmed barramundi (*Lates calcarifer*) and treatment of *Gyrodactylus turnbulli* on guppies (*Poecilia reticulata*) (Fridman *et al*., 2014).

Snubnose pompano (*T. blochii*), is considered a potential marine fish species for aquaculture because of its fast growth rate, excellent meat quality, and suitability for cage culture (Ma *et al*., 2014). Currently, sea lice infestation belonging to family Caligidae is the most significant disease problem affecting pompano, *T. blochii* reared in cages and tanks (Pakingking *et al*., 2018; Reyes *et al*., 2014; Cruz-Lacierda *et al*., 2011). In a study by Cruz-Lacierda *et al*. (2011), parasitic caligid identified as *Lepeophtheirus spinifer* caused lesions on the body surface and heavy infestation can cause high or mass mortality of *T. blochii*.

There have been growing concerns regarding the use of chemicals in parasite control due to reports of reduced efficacy and its effect on inducing resistance to fish pathogens when used inappropriately (Lee and Gao, 2012; Yanong, 2008). Malachite green and formalin are examples of conventional treatments for parasite infestation but can possibly be more toxic to the hosts rather than their parasites (Schelkle *et al*., 2013). An alternative way to solve this problem is to use medicinal or herbal plants like garlic (*A. sativum*). There are many reports about the antiparasitic activities of garlic in fish species (Ankri and Mirelman, 1999; Madsen *et al*. 2000; Buchmann *et al*., 2003). However, none of these previous studies have evaluated the efficacy of garlic extract in terms of the acute toxicity of pompano. Thus, the present study was conducted to determine the 96 h median lethal concentration (LC50) of aqueous garlic extract for pompano juveniles.

Materials and methods

Experimental set-up

Pompano juvenile (BW=138.8±41.1 g, TL=19.5±1.51 cm) obtained from Marine Hatchery (SEAFDEC/AQD), Tigbauan, Iloilo, Philippines were acclimatized for at least 2 weeks under laboratory conditions (salinity = 30 ppt, temperature = 280C). Fish were placed in a 500-L fiberglass tank provided with flow-through seawater system and gentle aeration. Fish were fed daily at 3 % body weight with feed for pompano formulated by SEAFDEC/AQD until use for the experiment.

Preparation of aqueous garlic extract

A 3 L stock solution at 1000 ppm garlic extract was prepared by dissolving 3 g of powdered garlic extract (Hebei Kangdali Pharmaceutical Co., Ltd., 25 % allicin) in 3 L distilled water. All concentrations were set using the formula: M1V1=M2V2.

Acute toxicity (LC_{50}) experiment

Two trials each of 96 h static bioassay tests were conducted at the Fish Health Wet Laboratory of SEAFDEC following standard procedures for toxicity tests outlined by the APHA-AWWA-WEF (2012). A completely randomized design was used for experiments with 10 fish per 20 L of seawater in glass aquaria provided with aeration. After a 1 hr acclimatization period (28 °C; 32 ppt salinity; 7.8 pH), six test concentrations were prepared (2.5, 5.0, 7.5, 10, 20 and 30 ppm) by adding the necessary volume of stock solution to the aquaria (**Table 1**). For the control, only seawater was used. All test concentrations including the control were done in three replicates per trial. Test fish were not fed during the entire experimental period.

Behavioral patterns of fish

Fish were observed every 24 h for four days (96 h) for behavioral changes and mortality. Moribund fish were removed and dissected immediately. Gills and liver of moribund fish were collected for histological analysis. Dead fish were removed, recorded and properly disposed. Water quality monitoring

Water quality parameters, i.e. salinity, temperature, dissolved oxygen, were measured and recorded daily. Temperature and salinity were measured using standard mercury thermometer and an AtagoS/Mill hand-held optical refractometer, respectively. Dissolved oxygen and pH were measured using digital Milwaukee MW 600 DO meter and Milwaukee MW 101 pH meter, respectively.

Histological analysis

The gills and liver samples from the control fish and exposed fish were subjected to histological processing. The gills and liver of *T. blochii* were dissected and fixed in Bouin's fixative. Tissues were processed, sectioned and stained with haematoxylin and eosin (Humason, 1979). Stained tissue samples were examined under a compound microscope.

Statistical analysis

Probit analysis program by Srinivasan (2004) based on Probit Analysis by Finney (1971) was used to calculate the median lethal concentration (LC50) values and corresponding 95 % confidence limits.

Results

Table 2 shows the values of water parameters measured during the 96 h of the experiment. The average temperature (28±0.0), salinity (32 ppt) and pH (8.0) were consistent until the last day of monitoring. However, the mean dissolved oxygen level decreased towards 96 h but the level was above 5 ppm.

In terms of behavior, test fish immersed in 10 to 30 ppm extract exhibited abnormal behavior in the first 24 h of the experiment. Fish became almost static upon addition of the said garlic extract concentrations to their respective aquaria. Test fish exposed to highest concentration (30 ppm) remained weak throughout the first 24 h, with frequent surface-to-bottom swimming and faster opercula activity were observed.

Table 1. Volume of stock solution added to experimental water for the different test concentration

Test concentrations (ppm)	Volume (ml) of stock solution (1000 ppm)
2.5	50
5	100
7.5	150
10	200
20	400
30	600

Table 2. Water parameter values (mean±SD) measured during the 96 h exposure of *T. blochii* to garlic extract (Hebei Kangdali Pharmaceutical C. Ltd., 25 % allicin) concentration

Time (h)	Temperature (⁰C)	Salinity (ppt)	pH	Dissolved oxygen (ppm)
0	28±0.0	32±0.0	8.04±0.01	9.0±0.2
24	28±0.0	32±0.0	7.12±0.02	8.66±0.2
48	28±0.0	32±0.0	7.86±0.02	6.6±0.2
72	28±0.0	32±0.0	7.83±0.02	6.0±0.2
96	28±0.0	32±0.0	7.83±0.02	5.0±0.3

Median lethal concentration or LC50 is an aqueous chemical activity which causes 50% mortality in the exposed population of fish. The 96 h LC50 test was conducted to measure the susceptibility and survival potential of fishes to a particular toxic substance. Higher LC50 value indicates lower toxicity because greater concentrations are required to cause 50% mortality in fishes. The cumulative mortality of snubnose pompano after exposure to garlic extract for 96 h was shown in **Figure 1**. The cumulative mortality rates were 0, 23.33 %, 53.33 %, 73.33 %, 86.66 % and 100 % at the concentrations of 2.5, 5.0, 7.5, 10, 20 and 30 ppm, respectively. No mortality was recorded in the control group. The 96 h LC50 was 7.48 ppm with lower and upper confidence limits of 6.19 ppm and 9.04 ppm respectively (**Table 3**).

Histopathological changes in the gills and liver were observed in 7.5, 10, 20 and 30 ppm. **Figure 2** shows the gills of pompano from the control group. At lower concentrations (2.5 and 5 ppm), gills appeared to be normal (**Figure 3**). Slight epithelial lifting and moderate hyperplasia occurred in fish treated with 10 and 7.5 ppm garlic extract after 96 h (**Figure 4**). Gill damage such as significant number of epithelial lifting, severe hyperplasia and fusion of the lamellae were noted in fish exposed to 20 ppm at 96 h and 30 ppm at 48 h (**Figure 5**). **Figure 6** shows liver cell morphology of the control group. Histopathological changes were not observed in lower concentrations (2.5 ppm and 5 ppm) (**Figure 7**). Fish exposed to 7.5 ppm and 10 ppm exhibited slight hypertrophy of the vacuolated-hepatocytes and sinusoidal dilation were also observed (**Figure 8**). Moreover, the frequency and intensity of change at 96 h of exposure was less than that of fish exposed

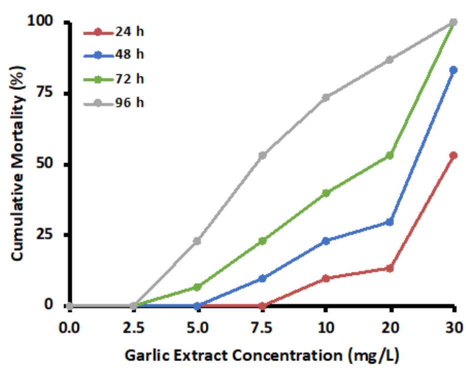

Figure 1. Percentage cumulative mortality of *T. blochii* exposed to different concentrations of garlic extract for various exposure time

Table 3. Computed lethal concentration (LC) values and confidence limits after 96h exposure of *T. blochii* juveniles (TL=19.5±1.51 cm) to garlic extract using Probit analysis.

Point	Probit	Concentration (ppm)	Log Concentration	95 % Confidence Limits	
				Lower	Upper
LC_1	2.67	0.60	-0.22	0.25	1.42
LC_{10}	3.72	1.65	0.22	0.95	2.86
LC_{50}	5.00	7.48	0.87	6.19	9.04
LC_{95}	6.64	28.43	1.45	17.78	45.47
LC_{99}	7.33	55.13	1.74	28.50	106.64

to higher concentrations (20 and 30 ppm). Blood congestion with hypertrophied vacuolated-hepatocytes, sinusoidal dilation and pyknosis were observed in 20 ppm at 96 h and 30 ppm garlic extract at 48 h (**Figure 9**).

Discussion

Measured temperatures were within the optimum range for *T. blochii*, which is 24-280C and optimum temperature is 27 °C (FAO, 2016). Salinity was also constant at 32 ppt throughout the experiment.

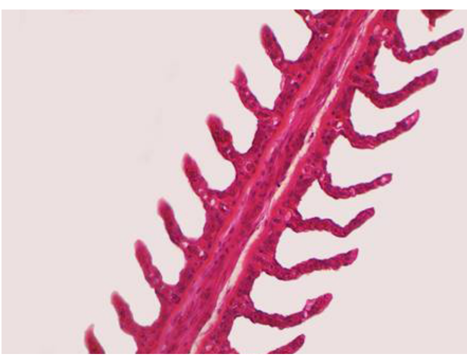

Figure 2. Section through the gills of pompano (*T. blochii*) of control group having no histopathological changes at 96 h. (H&E, 40x)

Salinity and pH values measured are within the optimum range for *T. blochii*, which are 5-40 ppt for salinity and 7.5-8.5 for pH (Jayakumar *et al*., 2013). According to Jayakumar *et al*. (2013), dissolved oxygen level for pompano should be maintained above 5 ppm.

T. blochii are highly active fishes and very sensitive to stimuli such as movements according to Reyes *et al*. (2014) thus it is considered an abnormal behavior for test fish to be static. Results pertaining to changes in behavior of *T. blochii* also agree with the findings of Syngai *et al*. (2016) wherein they also observed the carps gulping for air and having a static or slow swimming behavior after the addition of garlic aqueous solutions in the aquaria. In the study of Aqel *et al*. (1991), the garlic juice was tested *in vitro* to the isolated segments of aorta, trachea, intestine and heart of a rabbit. Their results imply that garlic juice has a relaxant effect on the smooth muscles of the rabbit. Acetylcholine is a neurotransmitter responsible for the action of muscles and it was inhibited by the addition of garlic juice. This evidence may be associated with the slow action and swimming behavior of test fish.

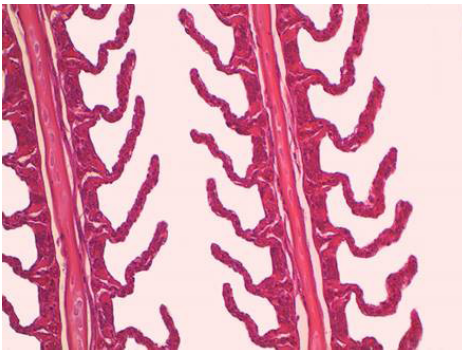

Figure 3. Section through the gills of pompano (*T. blochii*) exposed to 5 ppm having no histopathological changes at 96 h. (H&E, 40x)

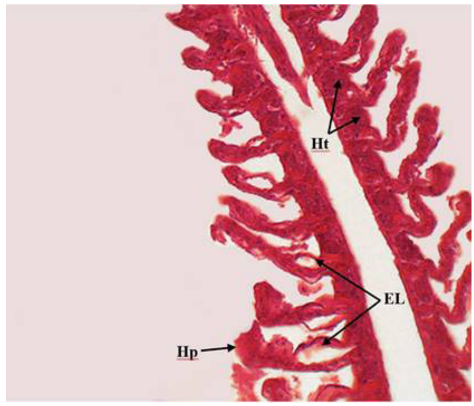

Figure 4. Section through the gills of pompano (*T. blochii*) exposed to 7.5 ppm garlic extract at 96 h showing slight epithelial lifting, moderate hyperplasia and hypertrophy. (H&E, 40x). EL=epithelial lifting, Hp=hyperplasia, Ht=hypertrophy

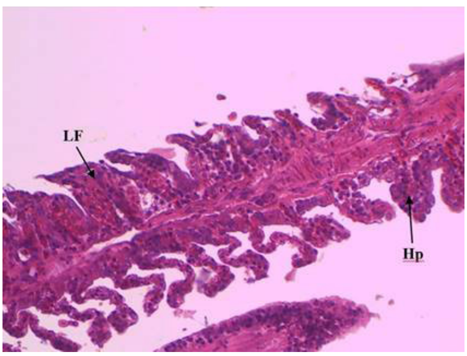

Figure 5. Section through the gills of pompano (*T. blochii*) exposed to 30 ppm garlic extract at 48 h showing severe hyperplasia and lamellar fusion of the secondary lamellae. (H&E, 40x). Hp=hyperplasia, LF=lamellar fusion

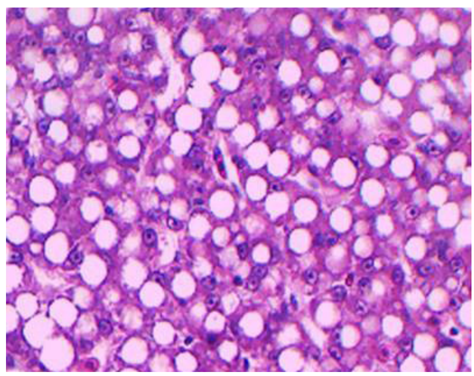

Figure 6. Section through the liver of pompano (*T. blochii*) of control group having no histopathological changes at 96 h. (H&E, 100x)

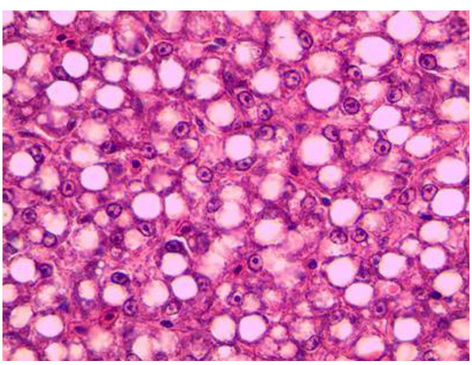

Figure 7. Section through the liver of pompano (*T. blochii*) exposed to 5 ppm having no histopathological changes at 96 h. (H&E, 100x)

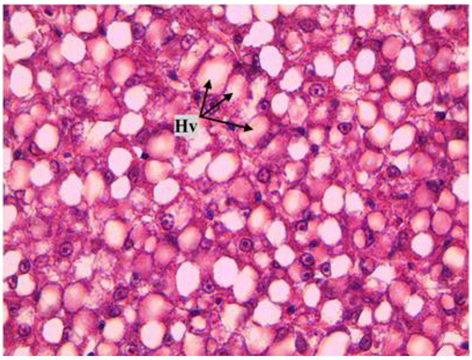

Figure 8. Section through the liver of pompano (*T. blochii*) exposed to 7.5 ppm garlic extract at 96 h showing slightly hypertrophied hepatocytes. (H&E, 100x). Hv=hypertrophied vacuolated hepatocytes

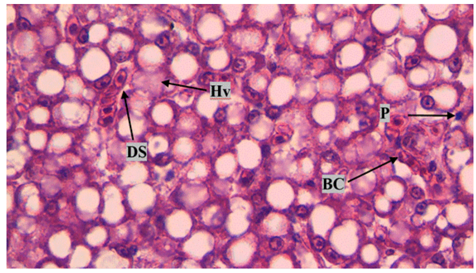

Figure 9. Section through the liver of pompano (*T. blochii*) exposed to 30 ppm garlic extract at 48 h showing blood congestion, dilated sinusoid, hypertrophied hepatocytes and pyknotic nuclei in some hepatocytes. (H&E, 100x). BC = blood congestion, DS = Dilated sinusoid, Hv = hypertrophied vacuolated hepatocytes, P = pyknosis.

Flores-Lopes and Thomaz (2011) reported that physiological responses of fish gills to irritants in water include inflammation, hyperplasia, lamellar fusion and epithelial lifting of the secondary lamellae. Similarly, *T. blochii* exposed to 7.5 to 30 ppm garlic extract showed varying degrees of epithelial lifting, hyperplasia and fusion of the secondary lamellae (Fig. 2). Epithelial lifting is one of the first pathological responses of the gills to pollutants by increasing the distance between the water and blood to block the entry of contaminants (Flores-Lopez & Thomaz, 2011). Lamellar fusion is also a defense mechanism of the fish against pollutants but limits gas exchange (Dane & Sisman, 2014).

Blood congestion, pyknosis, hypertrophied hepatocytes and sinusoidal dilation observed in the liver of *T. blochii* exposed to 7.5 to 30 ppm garlic extract are stress responses of fish to accumulation of toxicants (Kroemer *et al*., 2008). These histopathological changes were also recorded in the study of Al-Salahy & Mahmoud (2003) where they orally administered the garlic juice in Nile tilapia for 5 days and 11 days every 24 h and among the changes, cellular degeneration was the most abundant. Moreover, this is an indication that higher concentrations of garlic extract can induce histopathological changes in pompano. Supposedly, a normal hepatocyte is polygonal in shape with a centrally-located spherical nucleus. Vacuolation of the hepatocytes may be associated with high lipid diet of the fish prior to the experimental period. Hepatocytes oxidize fatty acids, however, in large quantities, hepatocytes are overwhelmed thus unable to oxidize the excess, causing fat deposition to occur. In the study of Fountoulaki *et al.* (2017), the lipid deposition in hepatocytes increased in meagre fish when fed with 20% lipid feeds. They observed steatosis or abnormal fat accumulation manifested by the vacuolation of hepocytes. However, Fountoulaki *et al.* (2017) implied that vacuolation of hepatocytes has no pathological consequences as the growth of test fish were consistently increasing over time. These changes also manifested in the liver of *Tor tambroides* fed with dietary linoleic and linoleic acid ratio where intense accumulation of lipid manifested as well (Ramezani-Fard *et al*., 2011). However, the pathological effects steatosis are not well studied. This fatty liver condition possibly is a result of an imbalanced diet and may be irreversible (Fountoulaki *et al*., 2017).

Fujisawa *et al*. (2008) have stated that allicin has low solubility in polar solvents like water, which may suggest that undissolved residues may have accumulated in the gills of *T. blochii* and reduced ionic and gas exchanges, thereby affecting its breathing. In the present study, water was also used as solvent for garlic extract because of the stability and longer chemical half-life of the active ingredient of garlic in water (Fujisawa *et al*., 2008). Similar findings due to accumulation of excess extracts were stated in a study by Claudiano *et al*. (2012), in which guppy (*Phalloceros caudimaculatus*) exposed to *Terminalia cattappa* extract showed difficulty in breathing. This further supports the possibility that the difficulty in breathing manifested in the surface-to-bottom swimming of *T. blochii* in the present study was due to the accumulation of undissolved garlic extract in gills.

Results in terms of mortality is in agreement with the study of Syngai *et al*. (2016) in common carp juveniles immersed in allicin where mortality rate also increased with increasing concentration of garlic extract. The present results further support the claim that lethality and susceptibility of *T. blochii* to garlic extract is concentration-dependent, as mortalities increased with increasing garlic extract concentration.

Conclusion

The findings of the present study suggest that aqueous garlic extract up to 5 ppm can be safely used in pompano for prophylactic purposes. The acute toxicity data obtained from this study provide baseline information on the dosage for the aqueous garlic extract to be used for future prophylaxis. *In vitro* and *in vivo* studies particularly on the efficacy of garlic extract in controlling and preventing parasitic diseases in various fishes are needed.

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