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RESEARCH ARTICLE

Acute lethal toxicity of dried garlic (*Allium sativum*) powder on orange-spotted grouper (*Epinephelus coioides*) juveniles under static exposure

G. Erazo-Pagador[1] [a], H.R. Dumaran-Paciente[1], B.J. Caloyloy[1]

[1] Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC/AQD), Iloilo, Philippines

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The present study was carried out to assess the toxic effect and determine the lethal concentration of dried garlic (*Allium sativum*) powder on the orange-spotted grouper (*Epinephelus coioides*) juveniles. Laboratory static bioassays were conducted to determine the 96-h LC_{50} of different concentrations of garlic (0, 30, 60, 90, 120, 150 and 180 mg L^{-1}) on grouper juveniles (mean weight, 35.4±6.18 g and mean length, 13.2±0.51 cm). The 96-h LC_{50} was 74.78 mg L^{-1} with lower and upper confidence limits of 62.8 and 89.06 mg L^{-1}, respectively. All fish exposed to 30 to 180 mg L^{-1} exhibited rapid movement, faster opercular activity and erratic swimming whereas at higher concentrations (90 mg L^{-1} to 180 mg L^{-1}) fish showed additional behavioral changes such as loss of balance, lethargy, gulping for air and respiratory distress. Water quality parameters were within the recommended acceptable limits. This LC_{50} value can be used as a baseline reference to generate an effective concentration of garlic for future prophylaxis and treatment for parasitic, viral and bacterial infection in grouper.

1. Introduction

Aquaculture is one of the most rapidly expanding animal culture systems and food-producing sectors, with total world fish production of 179 MT in 2018 (FAO, 2020). However, the intensification of the aquaculture industry has been associated with the increased occurrence of different fish diseases. Disease outbreaks continue to be a major constraint in aquaculture production as they can cause considerable economic losses in the industry (Foysal et al. 2019). Although effective therapeutants are available against diseases, these are primarily based on chemicals that are toxic to the environment and the host species (Palma, Cruz-Lacierda, and Corre 2015). Thus, there is a need for alternative treatments that are safe and environmentally friendly.

The utilisation of traditional plant-based medicine is an excellent alternative to manage aquatic animal diseases (Reverter et al. 2014; Valladão, Gallani, and Pilarski 2015; Van Hai 2015). One of these is garlic (*A. sativum*; Family Liliaceae) which is an edible plant and has strong prophylactic and therapeutic properties against bacteria (Feldberg et al. 1988; Ankri and Mirelman 1999; Corzo-Martinez, Corzo, and Villamiel 2007) and parasites (Rabinkov et al. 2000; Buchmann, Jensen, and Kruse 2003). It contains allicin, its major

a Corresponding author:
Email: gepagador@seafdec.org.ph

biologically active component (Lee and Gao 2012) and various compounds of amino acids that are highly unstable, volatile, cytotoxic, fat-soluble organosulfide compounds (Hussein, Hamdy, and Moussa 2013; Amagase et al. 2001). When garlic is crushed or chopped, the alliin (an amino acid derivative) interacts with the enzyme, alliinase to form allicin, giving the garlic its characteristics, odor and sulfur-containing compound. It was reported that garlic extract in bath treatment has anti-helminthic properties against *Capillaria* sp. in the common carp *(Cyprinus carpio*) at 200 mg L^{-1} (Peña, Auró, and Sumano 1988), and inhibitory effects for fish parasite infestation against *Ichthyopthirius multifiliis* (Buchmann, Jensen, and Kruse 2003) in Nile tilapia (*Oreochromis niloticus*) at 62.5 mg L^{-1} and *Neobenedenia* sp. (Militz et al. 2013) in farmed barramundi (*Lates calcarifer*) at 0.76 and 1.52 µL L^{-1}. It has also an antibacterial activity against *Aeromonas hydrophila* in rohu (*Labeo rohita*) when incorporated in feeds at 1 g kg^{-1} (Sahu et al. 2007) and in Nile tilapia (*O. niloticus*) (Aly and Mohamed 2010) at 30 g kg^{-1} and in Thai silver barb (*Barbonymus gonionotus*) at 8 mg mL^{-1} (Rahman et al. 2009).

The orange-spotted grouper (*Epinephelus coioides*) is one of the most commercially and economically important species of marine fish cultured in Southeast Asia including the Philippines (Marte 2002). The high price and strong market demand for groupers have driven small-scale farming, providing a livelihood for fishermen in coastal communities in the region (Sadovy de Mitcheson et al. 2012; Amorim et al. 2019). Groupers are commonly cultured in floating net cages in marine and estuarine environments as well as in brackishwater earthen ponds. Like other species farmed in these systems, grouper production has been hampered by disease outbreaks due to bacterial, parasitic or fungal infections that often lead to mass mortalities (Sadovy 2020).

A number of studies have shown the potential use of garlic in aquaculture, however, only a few studies have investigated its potential for controlling diseases on groupers. As a basis for optimum therapeutic application, determining the toxicity and lethal concentrations of garlic powder on grouper is necessary. Thus, the objectives of the present study are to determine the 96-h median lethal concentration (LC_{50}) of garlic and the behavioral response of juvenile groupers (*E. coioides*).

2. Materials and Methods

2.1. Experimental set-up

Healthy hatchery-reared juvenile groupers (mean weight, 35.4±6.18 g and mean length, 13.2±0.51 cm) were purchased from a commercial hatchery at Sarangani, Philippines. The fish were acclimatised for at least 2 weeks under laboratory conditions (salinity = 33 ppt, temperature = 28°C). Fish were placed in a 500-L fiberglass tank provided with a flow-through seawater system and aeration. Fish were fed daily at 3% body weight with formulated feeds (SEAFDEC/AQD grouper diet) until use for the experiment.

2.2. Preparation of garlic solution

Garlic powder containing 25% allicin was obtained from Hebei Kangdali Pharmaceutical Co., Ltd. Garlic aqueous stock solution at 9,000 mg L^{-1} was prepared by adding 9 g powdered garlic in 1 L distilled water and stored in a dark bottle at room temperature.

2.3. Determination of median lethal garlic powder concentration (LC_{50}) experiment

Two trials of 96 h static bioassay tests were conducted at the Fish Health Wet Laboratory of SEAFDEC/AQD following standard procedures for toxicity tests outlined by the APHA-AWWA-WEF (2012). The randomised design consisted of a control and six concentrations (30, 60, 90, 120, 150 and 180 mg L^{-1}) of garlic powder and were done in triplicates having ten fish per aquarium for a total of 30 fish and three tanks per test concentration. All fish were stocked in a 20-L glass aquarium filled with seawater and aeration was provided for 96 h. All the grouper juveniles were starved for 24 h, a day before the treatment and throughout the experiment. Mortality was recorded at 24, 48, 72 and 96 h of exposure.

2.4. Water quality monitoring

At 24 h intervals, the water quality parameters, i.e. salinity, temperature, dissolved oxygen, were measured and recorded in all aquaria. Temperature and salinity were measured using a standard mercury thermometer and an AtagoS/Mill hand-held optical refractometer whereas dissolved oxygen and pH were measured using a digital Milwaukee MW 600 DO meter and Milwaukee MW 101 pH meter, respectively.

2.5. Behavioral patterns of fish

Fish were observed every 24 h for four days (96 h) for behavioral changes and mortality. No water change was done throughout the experiment. Fish was considered dead if the opercular and tail movements stopped and if there was no response to gentle prodding. Dead fish were removed immediately from the aquarium to avoid fouling, recorded and properly disposed.

2.6. Statistical analysis

The LC_{50} values were used to determine the acute toxicity effect using the Probit analysis program by Srinivasan (2004) based on Probit Analysis by Finney (1971).

3. Results

3.1. Toxicity of garlic to juvenile groupers

Figure 1 shows the cumulative mortality of grouper juveniles after they were exposed to different concentrations of garlic powder for 96 h. Results showed that the cumulative mortality for 30, 60, 90, 120, 150, and 180 mg L^{-1} were 0, 36.66, 60.00, 76.66, 93.33 and 100%, respectively. On the other hand, there

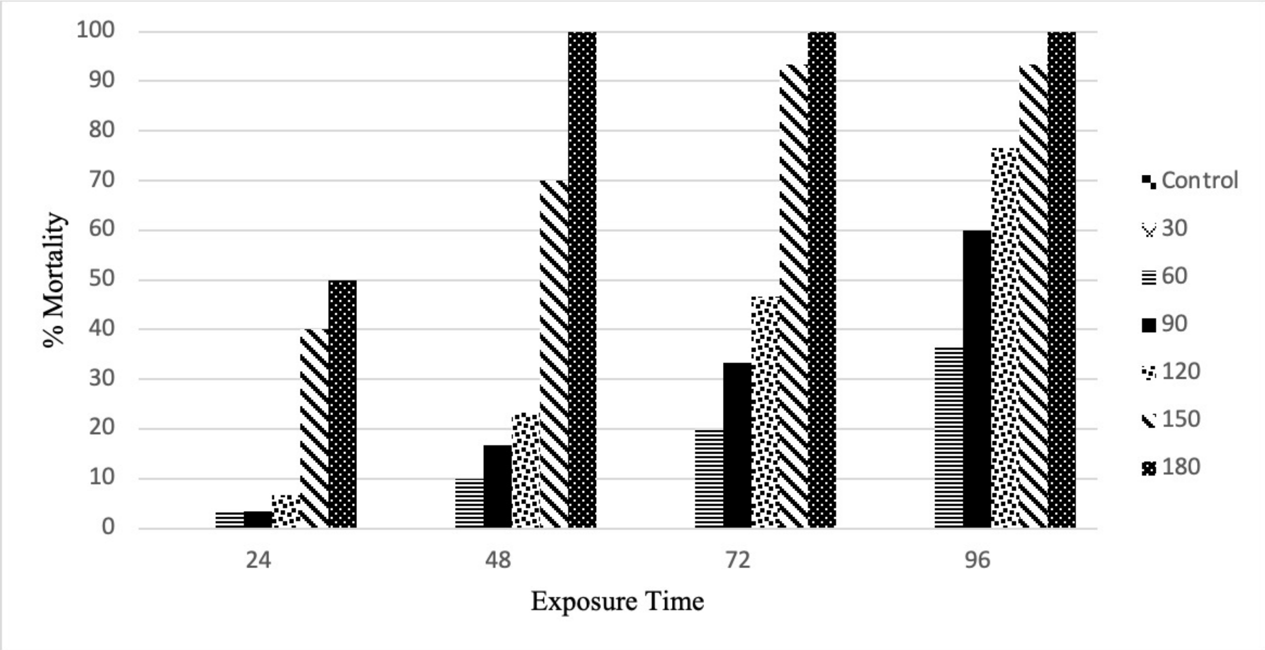

Figure 1. Cumulative mortality of *E. coioides* exposed to different concentrations (mg L^{-1}) of garlic powder for various exposure time.

Table 1. Lethal concentration (LC) values and confidence limits after 96 h exposure of *E. coioides* juveniles (BW=35.4±6.18 g; TL=13.2±0.51 cm) to garlic powder computed through Probit analysis.

Point	Probit	Concentration (ppm)	Log Concentration	95% Confidence limits	
				Lower	Upper
LC_1	2.67	20.56	1.31	10.44	40.49
LC_{10}	3.72	36.72	1.56	23.76	56.76
LC_{50}	5.00	74.78	1.87	62.8	89.06
LC_{95}	6.64	186.32	2.27	135.1	256.96
LC_{99}	7.33	271.98	2.43	169.3	436.93

was no mortality among the control group. However, 100% cumulative mortality was observed at the highest concentration tested. The median lethal (LC_{50}) garlic powder concentrations were determined as 184.45, 121.42, 103.02 and 74.78 mg L^{-1} garlic powder at 24, 48, 72 and 96 h of exposure, respectively. The 96-h LC_{50} of *E. coioides* exposed to various concentrations of garlic powder was 74.78 mg L^{-1} with lower and upper confidence limits of 62.80 and 89.06 mg L^{-1}, respectively (Table 1).

The regression equation was calculated to be Probit y = –13.131 + 9.1294 log Conc. X and on R square value (R^2) of 0.93 (Figure 2). These expressions, that is, the regression equation R^2 value indicated that the mortality rate of the test fish and concentrations of garlic powder are positively correlated. This means that the mortality rate of the fish increased with an increase in the concentration of garlic powder.

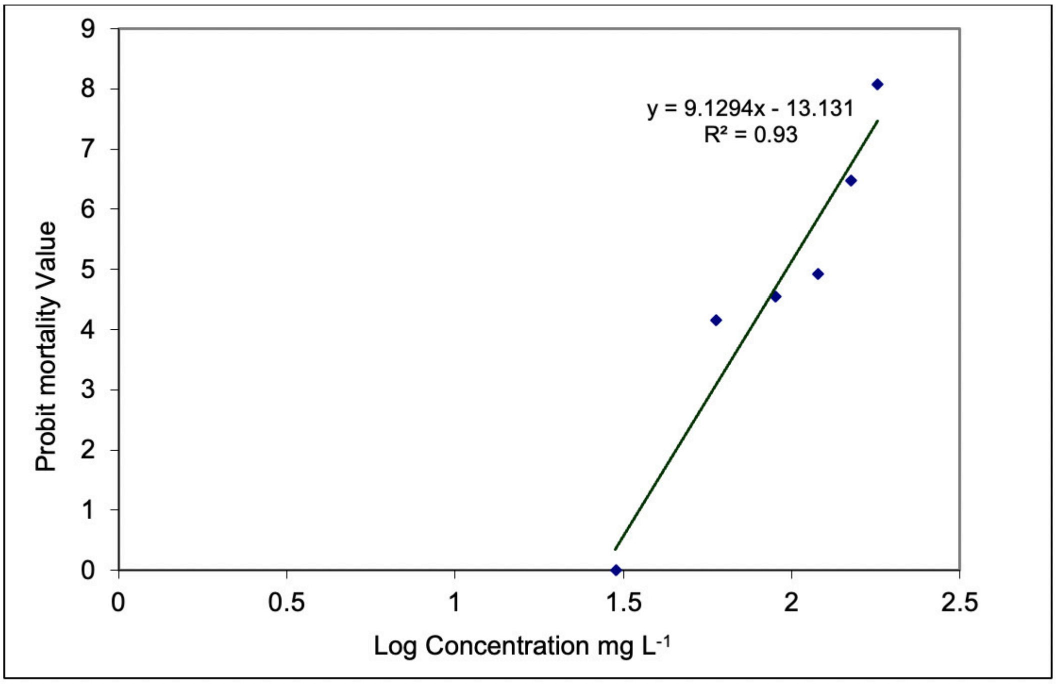

Figure 2. Linear relationship between mean probit mortality and log concentration of *E. coioides* exposed to various concentrations of *A. sativum* garlic powder for 96 h.

Table 2. Water parameter values (mean±SD) of all test concentrations during the 96 h exposure of *E. coioides* to powdered garlic (Hebei Kangdali Pharmaceutical C. Ltd., 25% allicin).

Time (h)	Temperature (^{0}C)	Salinity (ppt)	pH	Dissolved oxygen (ppm)
0	27.5±3.54	34.15±2.12	7.5±0.08	6.1±0.35
24	27.6±2.19	34.28±2.83	7.9±0.08	6.0±0.06
48	27.7±0.92	34.87±1.41	7.8±0.11	6.3±0.17
72	27.8±0.35	33.08±2.12	7.9±0.06	6.6±0.24
96	27.4±1.48	34.23±0.71	8.0±0.11	6.6±0.78

The mortality during 24, 48, 72 and 96 h exposure to garlic followed a concentration and time-dependent pattern where it increased at higher concentrations and longer exposure time (Figure 1). The garlic was found to be lethal on the second day of the experiment within 48 h at the highest concentrations of 180 mg L^{-1} wherein all fish were found to have died.

3.2. Water quality

Table 2 shows the mean values of water parameters of all test concentrations during the 96 h of the experiment. Temperature ranges from 37.4 to 37.8 °C, normal seawater salinity was maintained, DO levels were above 6 mg L^{-1} and pH slightly increased from 7.5 to 8.0 towards the last day of monitoring. The values of the water quality are within acceptable limits.

Table 3. Behavioral changes of *E. coioides* exposed to various concentrations of *A. sativum* powder at 24 h.

	Control	30	60	90	120	150	180
Rapid movement	-	+	+	+	+	+	+
Faster opercular activity	-	+	+	+	+	+	+
Erratic swimming	-	+	+	+	+	+	+
Loss of balance	-	-	-	+	+	+	+
Respiratory distress	-	-	-	-	+	+	+
Lethargy	-	-	-	+	+	+	+
Gulping for air	-	-	-	+	+	+	+

[1] (+) – shows behaviorial change
[2] (-) – not showing behavioral change

3.3. Behavioral response of groupers

The test fish exposed to different garlic concentrations during 96 h of exposure exhibited various behavioral patterns (Table 3). During the exposure time, fish at all tested concentrations showed rapid movement, faster opercular activity, erratic swimming behavior and fish settled down at the bottom of aquaria. It was observed that fish exposed to the highest concentrations (150 and 180 mg L^{-1}) of garlic powder exhibited behavioral responses such as loss of balance, respiratory distress, lethargy and crowding on the water surface. However, these observations were minimal in the group exposed to 30-90 mg L^{-1} of the garlic powder whereas behavioral changes were not observed in the control group. All altered behaviors were observed only for the first 24 h and consequently, fish were either dead or recuperated.

4. Discussion

The present study investigated the toxicity of garlic powder in juvenile groupers as the basis for its application as a safe alternative treatment against disease infections in this economically important species. During the static bioassay test, the values of water parameters were within the recommended range for grouper culture (APEC/SEAFDEC, 2001). The stability of water quality during the bioassay is critical in the accuracy of lethal concentrations derived in this study (Johnson and Finley 1980).

The LC_{50} or median acute toxicity test determines the relative toxicity of a particular chemical or substance to various species by providing estimates of the exposure concentration causing 50% mortality in the population of the test organism at a particular time (Johnson and Finley 1980). It is a general procedure for toxicity aquaculture studies to conduct LC_{50} testing because it allows complete chemical equilibrium between the fish and the test water (Syngai, Dey, and Bharali 2016). In the present study, the 96-h LC_{50} of garlic to juvenile grouper has been determined to be 74.78 mg L^{-1}. This value is considerably lower than 253.19 mg L^{-1} which is the reported 96-h LC_{50} of aqueous garlic extract to juvenile common carp (*C. carpio*) (Syngai, Dey, and Bharali 2016). The marked difference in the LC_{50} values can be attributed to the biological differences between the two species particularly in terms of detoxification and osmoregulation (Yeşilbudak and Erdem 2014). Findings from the present study further show the dose-dependent toxicity of *A. sativum* which is in agreement with other studies. For example, in the preliminary trials for acute toxicity of garlic to guppies of Fridman, Sinai, and Zilberg (2014), the survival rate was initially 30% when the fish were exposed to 20 ml L^{-1} for 1 h and increased to 100% after the concentration of garlic was adjusted to 12.5 ml L^{-1} for 1 h. It should be noted that LC_{50} values are species-dependent and even among conspecifics, the size, weight, sex and biological behavior further contribute to the variation (Tiwari et al. 2011). Another factor influencing the tolerance of fish to garlic is its size. Generally, larger fish can tolerate higher concentrations of chemicals and substances than smaller fish of the same species. This was evident in the study of Alam and Maughan (1995) wherein they exposed two group sizes, 3.5 and 6 cm, of carp (*C. caprio*) to mercury, lead, copper and nickel and found that LC_{50} value was significantly higher in larger fish. Grouper, in the current study, had an average size of 35 g while Fridman, Sinai, and Zilberg (2014) used 0.4 g guppy fish which has a significant weight difference and explains the higher survival in grouper. Additionally, the guppy fish (*Poecilia reticulate*) has a maximum tolerance of 12.5 ml L^{-1} aqueous garlic when immersed for 1 h which is lower than grouper (Fridman, Sinai, and Zilberg 2014). However, their exposure time is inconsistent with the experimental duration of the present study. No studies to date have investigated the acute toxicity of garlic to grouper thus comparison of sizes of conspecifics is not possible. Further studies on the toxicity of garlic to various sizes of groupers are necessary given that disease infections occur in all stages of grouper culture, from larvae to juveniles and as well as in broodstock. Moreover, higher LC_{50} values indicate more tolerance of fish to the experimental substance. This means that juvenile grouper have less tolerance when exposed to garlic as compared to juvenile common carp.

Changes in the behavior of fish can be assessed for determining the potential toxicity of a substance added to ambient water (Drummond and Russom 1990; Israeli-Weinstein and Kimmel 1998). Behavioral changes in the results were consistent with those reported by Syngai, Dey, and Bharali (2016) who also used the same technique to evaluate the toxicity of garlic to common carp *C. carpio* and they observed behaviors such as slow swimming, decreased response to external stimuli and faster opercular activity. Slow swimming behavior, lethargy, loss of balance and weak response to a stimulus can be associated with the inhibition of acetylcholine as garlic juice was found to have a relaxant effect on the smooth muscles such as the intestines, aorta and heart according to the results of Aqel, Gharaibah, and Salhab (1991). Additionally, the fish surfaces on the water and gulps for air to alternatively collect oxygen to compensate for the energy demand and erratically swims in the aquaria. These reactions may be an avoidant instinct as fish attempts to avoid contact with the toxicant (Kaur and Dua 2014; Patil and David 2008). Faster opercular movement may indicate a deficiency in oxygen intake which may be a result of hyperplasia, hypertrophy and aneurysm of the gills that limit the gas exchange

(Tiwari et al. 2011). Shivakumar & David (2004) mentioned that this behavior compensates for increased physiological activities in the exposure to toxic conditions.

This paper is the first to report the toxicity of garlic on grouper particularly the lethal concentration values, and behavioral effects, which are critical information to enable the application of garlic against grouper diseases. It has been reported that garlic can effectively eliminate *Ichthyopthirius*, a ubiquitous protozoan parasite in fish, at 62.5 mg L^{-1} (Buchmann, Jensen, and Kruse 2003). Taken together, this shows the strong potential of garlic to treat diseases in groupers and thus should be further investigated.

5. Conclusion

The LC_{50} of garlic *Allium sativum* powder to orange-spotted grouper *E. coioides* juveniles is 74.78 mg L^{-1}. This can serve as a baseline value for comparing toxicity bioassays of *A. sativum* to other species and the data can be used for future alternative therapy for fish diseases. It is suggested that ammonia levels must be included in the water parameters which may also have been attributed to the deaths of fish.

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