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Short Communication

In-vivo Toxicity Assessment of the Garlic Juice Extract (*Allium sativum*) in Juvenile Hybrid Grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*)

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ABSTRACT

The toxicity of garlic juice extract in juvenile hybrid grouper was evaluated via bath and oral administration. A total of 280 fish, each with an average weight of 20 ± 5 g, were evenly distributed among 28 glass aquaria. This distribution was designed to represent seven test concentration groups, each implemented in duplicate. The fish were immersed in freshly prepared garlic juice extracts at 0, 500, 600, 700, 800, 900, and 1,000 ppm concentrations. Meanwhile, pellets containing 0, 20, 40, 60, 80, and 100% garlic juice extract were administered for oral exposure. The median lethal concentration of garlic juice extract following bath immersion was recorded at 993.11 ppm after 96 hr. Besides, there was no mortality in all groups exposed to garlic juice extract orally, indicating that the extract has a shallow effect on juvenile hybrid groupers when ingested.

Keywords: *Allium sativum*, fish, garlic, hybrid grouper, toxicity

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INTRODUCTION

Garlic (*Allium sativum*) belongs to the Alliaceae family, with approximately 600 identified species of *Allium*. Its native range includes Europe, North America,

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North Africa, and Asia (Chen et al., 2019). Garlic has medical properties in addition to being widely used in cooking as a spicy and nutty flavour profile. Moreover, the predated ancient Egypt used it to treat illnesses among pyramid labourers, and it was also utilised for healing by the Babylonians, Greeks, and Romans (Koch & Lawson, 1996). In his earliest work in 1958, Louis Pasteur provided evidence of garlic's antibacterial properties (Bayan et al., 2014). Garlic extracts effectively treat stomach-related illnesses caused by common pathogenic intestinal bacteria and protozoan parasite *Entamoeba histolytica* (Shasany et al., 2000). Garlic oil inhalations are beneficial against coughs, red eyes, and impotence (Farid et al., 2022). Additionally, garlic has been shown to have anti-cancer effects and to reduce cardiovascular risk through its cholesterol-regulating properties (Zhang et al., 2020).

Petropoulos et al. (2018) previously reported that garlic bulbs are nutritionally dense in carbs and proteins. Garlic contains 33 sulphur compounds, 17 amino acids, many enzymes, and minerals. Its pungent odour is derived from alliin, a sulphur-containing molecule. When garlic is crushed or sliced, alliin (S-allyl cysteine sulfoxide) will be converted to allicin upon activating the alliinase enzyme (diallyl disulfide). Previous research has shown that allicin, a volatile sulphur compound, is responsible for a variety of intriguing activities such as antimicrobial, antioxidant, antihelminthic, immunostimulant, growth, and appetite stimulator (Fridman et al., 2014; Guo et al., 2015; Syngai et al., 2016).

A toxicity test is required before certain chemicals or treatments are intended for living organisms. Toxicity testing was described by Arome and Chinedu (2013) as evaluating and classifying the potential risks associated with a given test material. Acute aquatic toxicity would usually be determined using a median lethal concentration 96-hr (LC_{50}-96 hr) test, which quantifies the dose or amount of test compound capable of causing 50% mortality in a population following 96 hr of exposure (Islam et al., 2021). Various exposure modes, including oral and cutaneous routes, are utilised to determine the appropriate dosage for long-term toxicity studies (Strickland et al., 2018). A probable toxic effect can be detected through behavioural anomalies, typically reflected as a sign of damage to the nervous system upon exposure (Almeida et al., 2009).

The hybrid grouper is one of Malaysia's most cultivated marine fish species (Ridzuan et al., 2022), with a cumulative production of 3,871.88 metric tonnes in 2022 (Department of Fisheries [DOF], 2022). The species results from a crossbreeding program held by the Borneo Marine Research Institute (BMRI) and collaborators. Since then, the fish has gained popularity in the mariculture industry, most notably in Southeast Asia. The hybrid grouper is relatively easy to handle, has a high salinity and pH tolerance, and, perhaps above all, grows rapidly (Sutthinon et al., 2014). However, hybrid groupers, similar to other grouper species, are particularly prone to vibriosis, which results in severe mortality, especially at

the juvenile stage (Mohamad et al., 2019). Utilising a medicinal plant extract with proven antibacterial activity may reduce the impact. A previous study by Mercy and Gopalakannan (2018) reported that the aqueous preparation of garlic extract resulted in a remarkable inhibition of *Vibrio* sp. isolated from shrimp farms and, therefore, could be used to combat vibriosis. Valenzuela-Gutiérrez et al. (2021) reported multiple properties of garlic, including antibacterial and has been used in various aquaculture fish species such as tilapia, rainbow trout, spotted grouper, catfish and barramundi

However, studies on the toxicity effect of garlic juice extract in marine fish, especially hybrid grouper, are scarce. Hence, this paper reports a LC_{50}-96 hr of garlic (*A. sativum* L.) juice extract in juvenile hybrid grouper following bath immersion and oral ingestion exposure.

MATERIALS AND METHODS

Animal Ethics

The procedures for handling fish have adhered to the guidelines established by the Institutional Animal Care and Use Committee (IACUC), International Islamic University of Malaysia (IIUM), as certified in IACUC-2019-31.

Experimental Animals

The study procured healthy juvenile hybrid grouper with an average weight of 20 ± 5 g from the Marine Fishes Aquaculture Research Division, Fisheries Research Institute (FRI), Tanjung Demong, Terengganu, Malaysia. These fish were acclimatised in a 1 m^3 fibreglass tank. Subsequently, ten fish were slain and screened for bacterial, viral, and parasitic diseases. If any pathogen is identified, a treatment procedure will be implemented to ensure all the experimental fish are disease-free at the commencement of the trial. The fish were fed *ad libitum* with commercial feed, and the water was kept aerated continuously. Daily, the water was fully exchanged, and water quality parameters, including salinity, temperature, pH, and dissolved oxygen (DO), were determined with a handheld ProQuatro multiparameter (YSI, USA).

Extraction of Garlic Juice Extract

Three kilograms of fresh garlic (*A. sativum*) were purchased locally. The juice was extracted using an extractor by crushing peeled garlic bulbs. The stock extract was then subjected to filtration through a muslin cloth and subsequently stored in hermetically sealed bottles at 4°C until it was utilised. The juice collected was regarded as 100% garlic juice extract.

Preparation of Garlic Juice Extract for Bath Immersion Treatment

The freshly prepared garlic juice extract was diluted in seawater to achieve the desired concentrations of 500, 600, 700, 800, 900, and 1,000 ppm, following the methodology outlined by Yunus et al. (2019) with slight modifications.

Preparation of Garlic Juice Extract for Oral Exposure

The garlic juice extract was diluted in ethanol to achieve concentrations of 20, 40, 60, 80, and 100%, and subsequently sprayed onto fish pellets at a 50 ml/kg ratio and allowed to dry at room temperature with continuous airflow (D.-H. Lee et al., 2012; Syngai et al., 2016).

Experimental Design

The acute toxicity tests, aimed at evaluating the LC_{50}-96 hr exposure by bath immersion, were carried out according to Yunus et al. (2019) with slight modifications. For a 96-hr exposure period, a static renewal bioassay system with constant aeration was established, consisting of six groups of garlic juice concentration gradients and one untreated control. Following a two-week acclimatisation period, 280 juvenile hybrid groupers were divided evenly in each test concentration of 500, 600, 700, 800, 900, and 1,000 ppm, along with an untreated control group glass aquarium (50 cm x 40 cm x 50 cm) in duplicate filled with 50 L of seawater. The untreated group received no garlic extract addition, which served as a negative control. Water quality parameters, including salinity, temperature, pH, and DO, were routinely monitored throughout the study using a ProQuatro multiparameter (YSI, USA). At a predetermined time each day, full (100%) water was exchanged, and test solutions were replenished to preserve the desirable concentration of test extract. Fish were fed twice daily with a total of 2% of the average body weight, with commercial feed containing 44% crude protein (SeaMaster, Vietnam). The behaviour of fish was thoroughly observed, and the number of mortalities was recorded at 24-, 48-, 72-, and 96-hr intervals.

Similarly, a static system was employed in the acute toxicity testing via oral feeding, comprising five groups of garlic juice concentration gradients and one untreated control. In this setup, 20 fish were divided in duplicate into each test concentration of 20, 40, 60, 80, and 100%, and the untreated control group glass aquarium filled with 50 L of seawater. Each treated group was fed twice daily with a total of up to 2% of the average body weight with commercial feed containing 44% crude protein (SeaMaster, Vietnam) added with a respective concentration of garlic juice extract, whereas the untreated negative control fish were fed with commercial pellets without garlic juice extract. At a predetermined time each day, 100% water was exchanged, and the behaviour of the fish was thoroughly observed. Mortalities were documented at 24-, 48-, 72-, and 96-hr intervals.

Data Analysis

The results are expressed as the mean ± SD. The DO statistical significance was evaluated using one-way analysis of variance (ANOVA), and means were separated by Tukey's test using SPSS (version 25). Significant differences were considered at $p<0.05$. A curve fitting was performed using an online tool, MyCurveFit, to calculate the median lethal concentration (LC_{50}) values.

RESULTS

Determination of LC_{50} by Bath Immersion

Table 1 shows the mortality rate of juvenile hybrid grouper exposed to various doses of garlic juice extracts. Initial mortality was only observed on day 2 (2.5%) and day 3 (2.5%) in the group of fish immersed in 900 and 700 ppm of garlic juice extract, respectively. However, on day 4 post-treatment, there was widespread mortality, with 23 fish deaths (57.5%) in the group immersed in 1,000 ppm garlic juice extract. In contrast, there was no mortality in the untreated control groups, 500, and 600 ppm groups, during the 96-hr experiment. As a result, the LC_{50} of juvenile hybrid grouper following bath immersion in garlic juice extract was calculated at 993.11 ppm (Figure 1).

Figure 2 summarises the DO value in all tested groups following the 96-hr trial. At the commencement of the experiment, there was no significant difference ($p>0.05$) in DO levels across the groups. However, after 24 hr of bath immersion exposure, the DO levels in the 600, 700, 800, 900, and 1,000 ppm treatment groups decreased significantly ($p<0.05$). This downward trend persisted until the end of the experiment. The lowest DO level was recorded at 5.7 ± 0.04 mg/L in the 1,000-ppm group at 72 hr post-exposure. Meanwhile, the control and 500 ppm groups showed no significant decrease in DO ($p>0.05$) throughout the experiment. The fish exposed to various concentrations of garlic juice extract exhibited changes in behaviour. In the first 24-hr period, the fish in the group exposed to 700, 800, 900, and 1,000 ppm became less aggressive. However, the fish in the negative control group exposed to low doses (500 and 600 ppm) showed normal behaviour. After 72 hr, fish exposed to the highest concentrations (1,000 ppm) increased their opercula activity and gasped for air at the water surface. The fish also exhibit anomalous behaviour, including lethargy, being less attentive, having a pale body, and sometimes swimming erratically.

Determination of LC_{50} by Oral Treatment

After being fed a commercial pellet containing various concentrations of garlic juice extract (20, 40, 60, 80, and 100%), no mortality was encountered in any treatment groups and untreated control. Additionally, the experimental fish remained active and did not exhibit clinical signs or visible abnormalities consistent with toxicity. However, there were observations of fish at high concentrations (900 and 1,000 ppm) experiencing instances of regurgitation of a small number of fish feed during the experiment. Yet, they continued to exhibit normal levels of activity. Consequently, the determination of the LC_{50} was not feasible. Figure 3 presents DO levels in all tested groups following the 96-hr trial. There was no significant difference ($p>0.05$) in DO levels between treated and untreated groups.

Table 1
Mortality rate within 96 hr of bath immersion exposure to varied doses of garlic juice extract

Group (ppm) \ Time (hr)	12	24	48	72	96	Number of mortality	Percentage (%)
Control	0	0	0	0	0	0	0
500	0	0	0	0	0	0	0
600	0	0	0	0	0	0	0
700	0	0	0	1	1	2	5
800	0	0	0	0	1	1	2.5
900	0	1	0	0	2	3	7.5
1,000	0	0	0	0	23	23	57.5

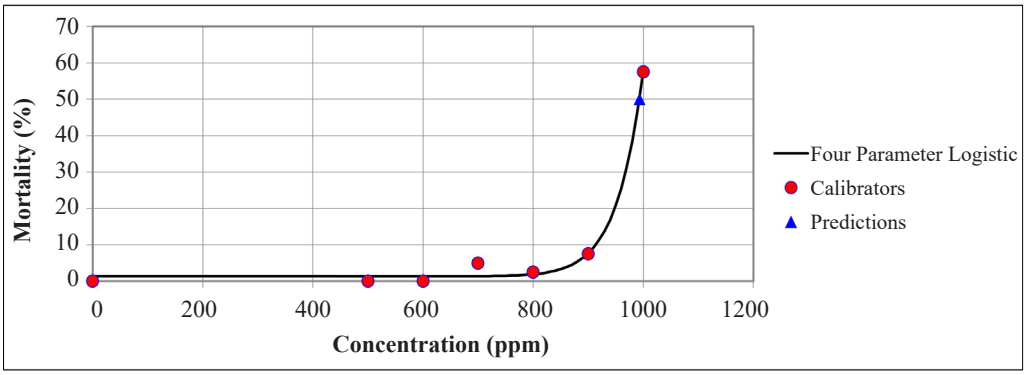

Figure 1. The median lethal concentration of garlic juice extract was calculated at 993.11 ppm following bath immersion within 96 hr

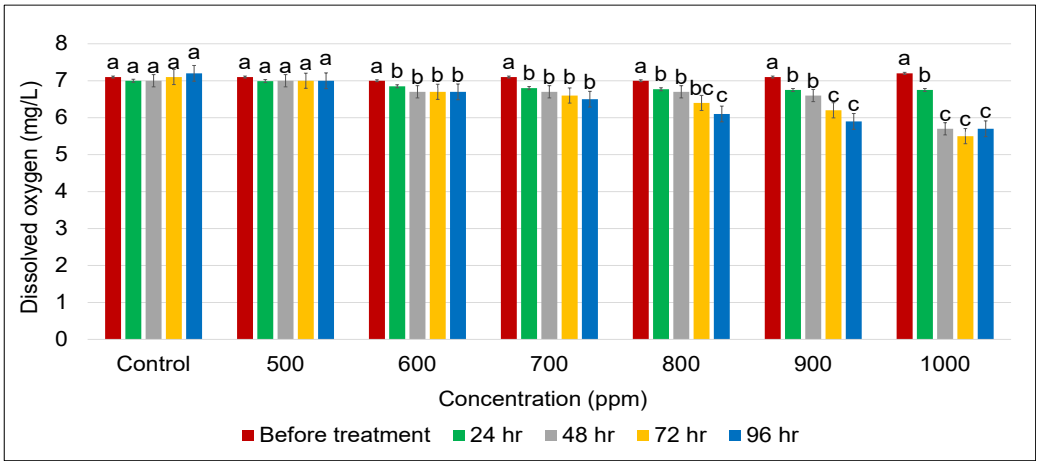

Figure 2. Values of dissolved oxygen in the seawater added with 500, 600, 700, 800, 900, and 1,000 ppm garlic juice extract. No garlic juice extract was added to the negative control group (Control)

Note. Different superscripts indicate a significant difference (p<0.05)

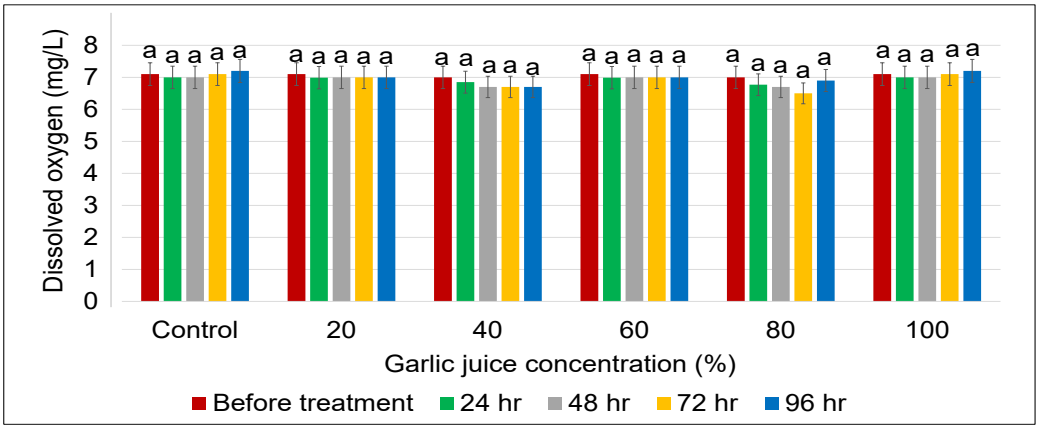

Figure 3. Values of dissolved oxygen in the seawater without adding garlic juice extract. However, juvenile hybrid groupers in those glass aquaria were fed commercial pellets with 500, 600, 700, 800, 900, and 1,000 ppm garlic juice extract. Juvenile hybrid groupers in the negative control group (Control) were fed with commercial pellet without garlic juice extract.

Note. Different superscripts indicate a significant difference (p<0.05)

DISCUSSION

The present study evaluates the acute toxicity of garlic juice extract in juvenile hybrid grouper by determining the LC_{50}, which represents the concentration of extract that caused 50% mortality after a continuous 96-hr exposure period. Therefore, the higher LC_{50} values reflect lower toxicity of the test substance, as greater concentrations are required to kill 50% of total organisms (Erhirhie et al., 2018). After bath immersion and exposure to garlic juice extract, this study obtained a high LC_{50} value of 993.11 ppm. Meanwhile, deaths occur in fish groups exposed to juice extract ranging from 700 to 1,000 ppm after 72 hr. This finding suggested that the susceptibility of the fish to the extract's toxicity effects was concentration- and time-dependent, with mortality rising as both factors increased.

It is believed that the cause of death was due to oxidised sulphur compounds toxicity because the DO level in all treatment groups was above 4 mg/L, the minimum requirement for grouper rearing (Nasukha et al., 2021). Allicin and other sulphur compounds in garlic juice extract are susceptible to oxidation, especially in the presence of water. The high dissolved oxygen in the water will react with these sulphur compounds, forming various sulphur-containing oxides such as sulphur dioxide (SO_2) and sulphur trioxide (SO_3). Both compounds are toxic and cause severe irritations to the respiratory tract, skin, and mucous membranes (Agency for Toxic Substances and Disease Registry [ATSDR], 2023a, 2023b). The operculum of the dead fish was wide open, indicating that the fish was battling for oxygen. This condition might be due to the damaged respiratory system from the toxicity of oxidised sulphur compounds. The higher concentration of garlic juice extract in the water will result

in a lower dissolved oxygen concentration due to the oxygen-sulphur reaction. The phenomenon was observed in the 1,000-ppm immersion group, where the significantly lowest concentration of DO was recorded. The lowest concentration of DO in the water indicated the highest concentration of toxic oxidised sulphur compounds in the water, which led to the highest mortality rate. The dead fish's body also turns pale and noticeably discoloured, owing to the garlic's burning effect and low oxygen level. A similar study indicated that the depigmentation of the fish body under toxic stress is associated with endocrine gland dysfunction (Herrera et al., 2019).

In contrast, the garlic-enriched feed ingests are exceptionally safe for juvenile hybrid grouper. After being fed with a varied concentration of garlic juice extracts, no mortality was encountered in any treatment groups. Furthermore, throughout the experimental trial, all fish remained highly active and showed no visible signs of toxicity. Therefore, the LC_{50} of fish fed with a commercial pellet containing varying percentages of garlic juice extract could not be determined. Garlic juice has been widely employed in other farmed animals for various purposes. As such, garlic aids in the digestion, absorption, and retention of nitrogen in cattle (Wanapat et al., 2008). Previous studies by Foysal et al. (2019) indicated that a variety of organosulfur compounds in garlic, such as S-methyl cysteine sulfoxide, diallyl disulfide, sallycysteine, ajoene, and allicin garlic, improve the performance tilapia's gut microflora which subsequently increases digestion, energy consumption, and promotes their growth. However, supplementing garlic as a feed additive should be done with caution. High concentrations of this plant extract may impair fish growth by facilitating excessive alkyl sulphide into the intestine, which could interfere with normal metabolism (J.-Y. Lee & Gao, 2012).

Assessing the toxicity of garlic juice extract across different fish species is vitally important for understanding its potential biological impact. Certain species might be more sensitive to garlic's sulphur and other bioactive compounds. Matthee et al. (2023) reported that freshwater fish species are usually more efficient in the uptake of drugs from the water compared to their seawater counterpart but will also depend on other factors such as chemical characteristics. This circumstance is because freshwater species absorb more water via their skin. On the other hand, something beneficial or harmless for one species could be detrimental or toxic to another. Hence, conducting toxicity tests on various fish species will help to understand the range of responses as well as potential differences in tolerance and susceptibility.

CONCLUSION

The recommended dose for immersion of garlic juice extract is 600 ppm or below. At concentrations of 700 ppm and above, fish started to die due to oxidised sulphur toxicity. However, the present finding has unveiled that garlic juice extract is safe to be used orally for hybrid grouper without

any toxicity effect. Therefore, the juice extract can be utilised as a prophylactic or treatment in aquaculture setup. The present data on acute toxicity will serve as a guideline for defining the recommended dosage of garlic juice extract in marine fish health management, especially for grouper species. However, other toxicity tests should be conducted in the freshwater fish species due to differences in biological behaviour, especially water and drug uptake levels via the skin.

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