ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 3, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

EVALUATION OF TOXICITY LEVELS OF THE AQUEOUS EXTRACT OF *ALLIUM SATIVUM* AND ITS EFFECTS ON THE BEHAVIOR OF JUVENILE COMMON CARP (*CYPRINUS CARPIO* L., 1758)

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Received: 16 March 2016, Revised and Accepted: 24 March 2016

ABSTRACT

Objectives: To evaluate the toxicity levels of the aqueous extract of *Allium sativum* on *Cyprinus carpio*.

Methods: Fresh garlic (*A. sativum*) was procured from a local market in Shillong, Meghalaya, India; and the crude aqueous extract of which was used in the acute toxicity tests to determine the 96 hrs median lethal concentration 50% (96 hrs LC_{50}) on juvenile common carp (*C. carpio*) via bath immersion treatment.

Results: The 96 hrs LC_{50} of the aqueous garlic extract for juveniles of *C. carpio* was estimated to be 253.19 mg/L. Furthermore, fish exposed to the different concentrations of the aqueous garlic extract exhibited abnormal behavior. The susceptibility of *C. carpio* to the lethal effects of the aqueous garlic extract was duration and concentration-dependent. Fish mortality was found to be associated with the decrease in pH values.

Conclusion: The current study indicates that the aqueous garlic extract has a low toxicity on *C. carpio* and therefore can be safely used in this species for any experimental purpose. Besides this, it also provides baseline information on the dosage for the aqueous garlic extract to be used as a potential management tool in aquaculture.

Keywords: Garlic, Lethal concentration 50%, Fish, Bath, Treatment.

INTRODUCTION

Allium sativum, commonly known as garlic, is a member of the family Alliaceae [1], and it has a history of dietary and medicinal applications for curing various diseases [2,3]. The medicinal effects of garlic are based on the organo-sulfur compounds, particularly allicin, which has potential antibacterial, antiprotozoal, antifungal, antiviral, antitumorigenic, antimutagenic, antioxidant, and detoxification properties [3,4]. The use of garlic in aquaculture has been reported to promote growth, stimulate appetite, provide a tonic to improve the immune system, provide antistress protection and can be a proven prophylactic and therapeutic agent [3]. On this aspect, Peña *et al.* [5] first reported the antihelminthic properties of crushed garlic against *Capillaria* sp. in the common carp (*Cyprinus carpio*), and more recently Fridman *et al.* [6] reported the *in vivo* efficacy of aqueous garlic extracts bath treatments against gyrodactylids infection in guppy (*Poecilia reticulata*). The study is a further complement to prior works on the *in vivo* efficacy of garlic-based bath treatments against gyrodactylids in the guppy (*P. reticulata*) reported by Schelkle *et al.* [7] and in the nile tilapia (*Oreochromis niloticus*) reported by Abd El-Galil and Aboelhadid [8].

Although garlic extracts have been reported to exhibit anticadmium toxicity properties in cell lines [4,9], to alleviate cadmium-induced oxidative stress in freshwater catfish (*Clarias batrachus*) [10], and to provide protection against cadmium-induced testicular oxidative damage and spermiotoxicity in rats [11], studies related to the application of the garlic extract on fishes as an antidote against heavy metal toxicity are still very scarce. Hence, further investigations are required to look at this important aspect. However, the use of certain xenobiotics in the aquatic system and food cycle can be made only after the acute toxicity tests, and biological dissociation tests have been carried out in detail [12].

Acute toxicity tests provide information on the time and/or concentration causing a significant effect or detectable response in 50% of the exposed population of test organisms. The tests are considered ecologically significant and legally defensible, simple, and cost effective. Acute toxicity studies can also provide fast and valuable information and indicate whether further toxicity studies should be conducted. Toxicity tests have traditionally been performed with a variety of fresh-water and salt-water species representing algae, fish, and invertebrates. Although the initial aquatic toxicity tests were carried out using bacteria, invertebrates, and other groups, they can in no way replace the actual tests performed on fish; which belongs to the last chain in the aquatic food cycle [13].

Common carp (*C. carpio* L., 1758) is one of the most important fish cultured in the world, either as food or for recreational fishing. Carp is often recommended for the baseline evaluation of emerging pollutants in aquatic ecosystems and commonly used in experimental models due to its availability and good adaptation to laboratory conditions [14].

Behavior changes, on the other hand, are the most sensitive indication of potential toxic effects. Optomotor responses are very useful in the evaluation of the behavioral changes of fish. Furthermore, behavioral changes are good indicators of damage to the central nervous system, as a consequence of exposure to toxic agents [12,15].

In general, plant extracts have been reported to favor various activities such as antistress, growth promotion, appetite stimulation, enhancement of tonicity and immune stimulation, maturation of culture species, aphrodisiac and antipathogen properties in fish, and shrimp aquaculture due to active principles such as alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids, or essential oils. So, their use could reduce costs of treatment and be more environmental-friendly as they tend to be more biodegradable than synthetic molecules. However, as the effect of plant products on fish is dose-dependent and there is also a potential for overdosing, so determining the suitable extract concentration is of great importance [16]. Consequently, there is a need

to first evaluate the toxicity levels of some of the herbal extracts on fishes so as to allow formulation of antidotes to counter the effects of some heavy metals and other pollutants. However, against this background and according to our knowledge; none of the previous studies have till now evaluated a bath immersion application of the aqueous garlic extract in terms of its acute toxicity on common carp. Hence, the purpose of this study was to evaluate through toxicity tests the 96 hrs median lethal concentration, 50% (LC_{50}) of the aqueous extract of garlic via bath immersion treatment and examine the accompanied behavioral changes in juvenile common carp (*C. carpio*) so as to provide baseline information.

METHODS

Preparation of aqueous garlic extract

Fresh garlic (*A. sativum*) was procured from a local market in Shillong, Meghalaya, India. The crude aqueous extract of garlic was prepared by grinding 10 g of peeled garlic cloves in 50 ml distilled water in a domestic blender [6]. The stock extract was then filtered through a muslin cloth and stored at 4°C in a sealed bottle. The extract was considered as the 100% stock solution and its concentration to be 200,000 mg/L. It was from this solution that the different static toxicity test doses were calculated and prepared by appropriate dilution.

Fish

Juvenile common carp (*C. carpio*) were procured from fish suppliers in and around Shillong, Meghalaya, India, and transported to the laboratory in oxygenated polyethylene bags. They were given prophylactic dip treatment in formalin (0.4%) and sodium chloride (0.7 g/l) for 15 minutes to keep away dermal infections [17,18]. The fishes were then held in well-aerated glass aquaria (40 cm × 120 cm × 40 cm) filled with 160 L of non-chlorinated tap water and allowed to acclimatize for 2 weeks to the laboratory conditions under a natural photoperiod 12 hrs:12 hrs (light:dark) cycle. During the acclimation period, the fishes were fed with a commercial fish feed (Tokyu, Thailand). No sex differentiation of the fishes was done while performing these studies.

Acute toxicity test

The experimental procedure for the acute toxicity tests to determine the 96 hrs LC_{50} of the aqueous garlic extract of *A. sativum* on *C. carpio* via bath immersion treatment was designed from the combination of methods described by Abel and Papoutsoglou [19], Thophon *et al.* [20], Zhao *et al.* [21], and Audu *et al.* [22]. A static renewal bioassay system consisting of five groups of concentration gradients and 1 blank control group with continuous aeration was set up for 96 hrs, and the water temperature was maintained as described by Tu *et al.* [23], using submersible thermostat controlled heaters (Xilong and Sobo, China). After 2 weeks of acclimatization, a total of 10 fish (4.06±0.05 cm standard length, 2.41±0.08 g weight) from the stock tank were distributed in each test concentration and control experimental glass aquaria (30.5 cm × 61 cm × 30.5 cm) filled with 30 L water. The 5 groups of fishes were exposed to different concentrations of 100, 200, 300, 400, and 500 mg/L of the freshly prepared aqueous garlic extract. For the blank control, distilled water was used. Aeration was stopped at the time of dosing [24]; and each group was assayed in triplicate. The fishes were starved 24 hrs before and during the experiment. Water quality monitoring was done at the start, during, and after the experiment by measuring the water temperature using a mercury glass thermometer graduated in degree Celsius (−10 to 110°C); dissolved oxygen (DO) using a digital DO meter (EUTECH Cyber Scan DO 110, Eutech Instruments Pvt. Ltd., Singapore); pH using a digital pH meter (Cyber Scan 1000 pH, Eutech Instruments Pvt. Ltd., Singapore); conductivity, total dissolved solids (TDSs) and salinity using a digital water analyzer (SYSTRONICS Water Analyzer 371, Systronics India Ltd., Ahmedabad, Gujarat, India). The water in the aquaria was replaced by half at a fixed time every day; during which time the test solutions were also renewed so as to maintain the concentration of the test extract. Fish behavior was closely observed and video recorded [15]. The number of deaths was also recorded at the intervals of 24, 48, 72, and 96 hrs. Dead fish were promptly removed, and the method to determine death in experimental fish was a lack of reaction on prodding the fish's tail with a glass bar [25].

Ethical note

The research undertaken was approved by the Institutional Ethics Committee (IEC) of Lady Keane College, Shillong, Meghalaya, India (No: C/16/LKC/IEC/2014/857); and it was performed as per the Organization for Economic Co-operation and Development (OECD) guidelines 203 [26], and it also adhered to the Animal Research: Reporting *In Vivo* Experiments guidelines produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research [27]. Furthermore, the experimental fish *C. Carpio,* which was used in the study, is a commercially important and not endangered fish and so the provisions of the Government of India's Wildlife Protection Act of 1972 are not applicable for an experiment on this fish.

Statistical analysis

The 96 hrs LC_{50} was computed according to Finney's Probit Analysis [28] using the EPA probit analysis program, version 1.5; and the "Trimmed Spearman-Karber method" [29] using the Phar Lap Inc., version 4.1L software (1986-92). Results were reported as mean±standard error.

RESULTS

Median LC_{50} determination

The number of fish deaths recorded at various concentrations of the extract and at different time intervals are shown in Table 1.

The relationship between the aqueous garlic extract concentration and the percentage mortality rate of *C. carpio* (L., 1758) at the end of the 96 hrs exposure period has also been indicated (Table 2).

No mortality was recorded in the control group. The results obtained from the 96 hrs static renewal acute toxicity experiments of the aqueous garlic extract on common carp, and the estimated LC_{50} values with their confidence limits have been listed in Table 3.

Chi-square for heterogeneity (calculated) = 1.159

Chi-square for heterogeneity (tabular value at 0.05 level) = 7.815

Mu = 2.403452

Sigma = 0.188263

Parameter	Estimate	SE	95% confidence limits
Intercept	−7.766474	3.211843	(−14.061686, −1.471262)
Slope	5.311723	1.304752	(2.754410, 7.869036)

Theoretical spontaneous response rate=0.0000. SE: Standard error

Table 1: Recorded number of fish deaths at various concentrations of the aqueous garlic extract and different exposure periods

Concentration (mg/L)	Number exposed	Mean fish deaths at 24 hrs	Mean fish deaths at 48 hrs	Mean fish deaths at 72 hrs	Mean fish deaths at 96 hrs
100	10	-	-	-	0.33
200	10	-	-	0.67	2.33
300	10	-	1.00	2.33	2.33
400	10	-	1.33	3.67	3.33
500	10	-	3.00	4.67	2.33

The 96 hrs LC_{50} of the aqueous garlic extract via bath immersion treatment on *C. carpio* was found to be 253.19 mg/L with the confidence interval ranging from 193.56 to 308.10 mg/L using the EPA computer program based on Finney's probit analysis method. This value was estimated to be 259.99 mg/L with the confidence interval ranging from 212.05 to 318.75 mg/L with the "Trimmed Spearman-Karber method." Fig. 1 shows the plot of Finney's adjusted probits and LC_{50} results.

Water quality analysis

During the toxicity tests, the recorded values for DO of the water in the aquaria were 6.08±0.05 mg/L; pH, 6.91±0.02; temperature, 20.08±0.02°C; conductivity, 110.06±1.01 µS/cm; TDSs, 70.72±0.81 mg/L; and salinity 0.07±0.001 parts per thousand, respectively (Table 4).

Behavioral patterns of fish

In the control tank, the experimental fishes exhibited normal behavior and swimming patterns without any mortality. The fishes exposed to different concentrations of the aqueous garlic extract, on the other hand, showed abnormal behavior which was observed only at 24 hrs after the addition of the fresh extract. The fishes became alert, stopped swimming, and remained static in position in response to the sudden changes in the surrounding environment. Frequent surface-to-bottom movements and faster opercula activity were observed as surfacing and gulping of air increased with the increase in the concentration of the extract. Similarly, it was also observed that the fishes remained in a vertical position for a few minutes with the anterior side or terminal mouth up near the surface of water trying to gulp air, and the tail was in a downward position. Soon, the fishes settled to the bottom of the tank and were found lying at the aquarium bed before they died.

Table 2: Relationship between the aqueous garlic extract concentration and the percentage mortality of *C. carpio* (L. 1758) at the 96 hrs exposure period

Concentration (mg/L)	Log concentration	Number of fish used	Mean number of total dead fish(96 hrs)	Mean mortality rate (%)
100	2.00	10	0.33	3.3
200	2.30	10	3.00	30.0
300	2.47	10	5.66	56.6
400	2.60	10	8.33	83.3
500	2.69	10	10.00	100.0

C. carpio: *Cyprinus carpio*

Table 3: Estimated LC values and confidence limits

Point	Exposure concentration	95% confidence limits	
		Lower	Upper
LC 1.00	92.363	31.336	137.802
LC 5.00	124.102	54.782	170.074
LC 10.00	145.270	73.536	190.914
LC 15.00	161.561	89.490	206.894
LC 50.00	253.193	193.556	308.098
LC 85.00	396.797	324.132	592.582
LC 90.00	441.296	354.442	714.672
LC 95.00	516.566	401.106	951.610
LC 99.00	694.078	498.669	1651.518

LC: Lethal concentration

DISCUSSION

LC_{50} is the ambient aqueous chemical activity that causes 50% mortality in an exposed population. These calculations are based on two important assumptions. The first assumption is that the exposure time associated with the specified LC_{50} is sufficient to allow almost complete chemical equilibration between the fish and the water. The second assumption is that the specified LC_{50} is the minimum LC_{50} that kills the fish during the associated exposure interval. Fortunately, most reliable LC_{50} satisfy these two assumptions.

The 96 hrs LC_{50} tests are conducted to measure the susceptibility and survival potential of organisms to particular toxic substances. Higher LC_{50} values indicate lower toxicity because greater concentrations are required to produce 50% mortality in organisms [30].

In this study, the acute toxicity effect of the aqueous garlic extract was studied in terms of the susceptibility, survival potential, and alterations in the behavioral patterns of the common carp (*C. carpio*). The susceptibility of *C. carpio* to the lethal effects of the aqueous garlic extract was duration- and concentration-dependent, as mortality increased with an increase in the concentration and exposure period; with the higher concentrations of 400 and 500 mg/L recording a high number of fish deaths at the 72 hrs exposure period itself (Tables 1 and 2). Similar findings have also been reported by Fafioye *et al.* [31] when *Clarias gariepinus* juveniles were exposed to the aqueous and ethanol extracts of *Parkia biglobosa*. In the current study, it is possible that the cause of fish mortality was associated with the decrease in pH values which was found to be inversely proportional to the increase in extract concentration; as pH influences toxicity by way of causing an electrolyte imbalance [32]. However, these findings disagreed with the work of Yunis Aguinaga *et al.* [33], who reported that fish mortality could not be attributed to pH; but instead were associated with the low levels of DO when the neotropical fish *Hyphessobrycon eques* was exposed to the aqueous extract of *Uncaria tomentosa* bark. In terms of the behavioral patterns, the observations in this study agreed with that of Lin and Liu [34], who reported that clinical signs such as abnormal movement and high respiration rate in hybrid tilapia (*Oreochromis mossambicus*) induced by ammonia suggested neurological dysfunction and gill damage. Likewise, the deficiency of oxygen causes the hypoxic condition in fish which results in an increase in the breathing rate and to cope with the condition, the fishes gulp air by frequent surfacing [35]. Furthermore, it is possible that the excess of extract gets accumulated in the gills, reducing gaseous and ionic exchanges [36]. The abnormal behavior, which was observed only at 24 hrs after the addition of the fresh extract agrees with the findings of Yunis Aguinaga *et al.* [33], who reported that after 24 hrs of exposure to the aqueous *U. tomentosa* plant extract, the fishes exhibited clinical and behavioral alterations.

Table 4: Values of DO, pH, temperature, conductivity, TDSs and salinity of the water; at the beginning, during and at the end of the toxicity tests with different aqueous garlic extract concentrations (0, 100, 200, 300, 400, and 500 mg/L)

Garlic concentration (mg/L)	DO (mg/L)	pH	Temperature (°C)	Conductivity (µS/cm)	TDS (mg/L)	Salinity (ppt)
0 (Control)	5.34-6.70	6.75-7.25	20-20.5	91.4-116	53.4-69.4	0.06-0.08
100	5.62-6.70	6.65-7.30	19.5-20.5	95.7-117	59.8-77.2	0.06-0.08
200	5.62-6.95	6.72-7.34	20-20.5	95-125	63.3-82	0.06-0.08
300	5.67-6.91	6.6-7.38	20-20.5	96.3-125	62.1-84.1	0.06-0.08
400	4.95-6.88	6.58-7.38	20-20.5	96.8-131	64.7-87.3	0.06-0.08
500	4.56-6.95	6.49-7.38	19.5-20.5	100-130	66.2-85.6	0.06-0.08

DO: Dissolved oxygen, TDSs: Total dissolved solid

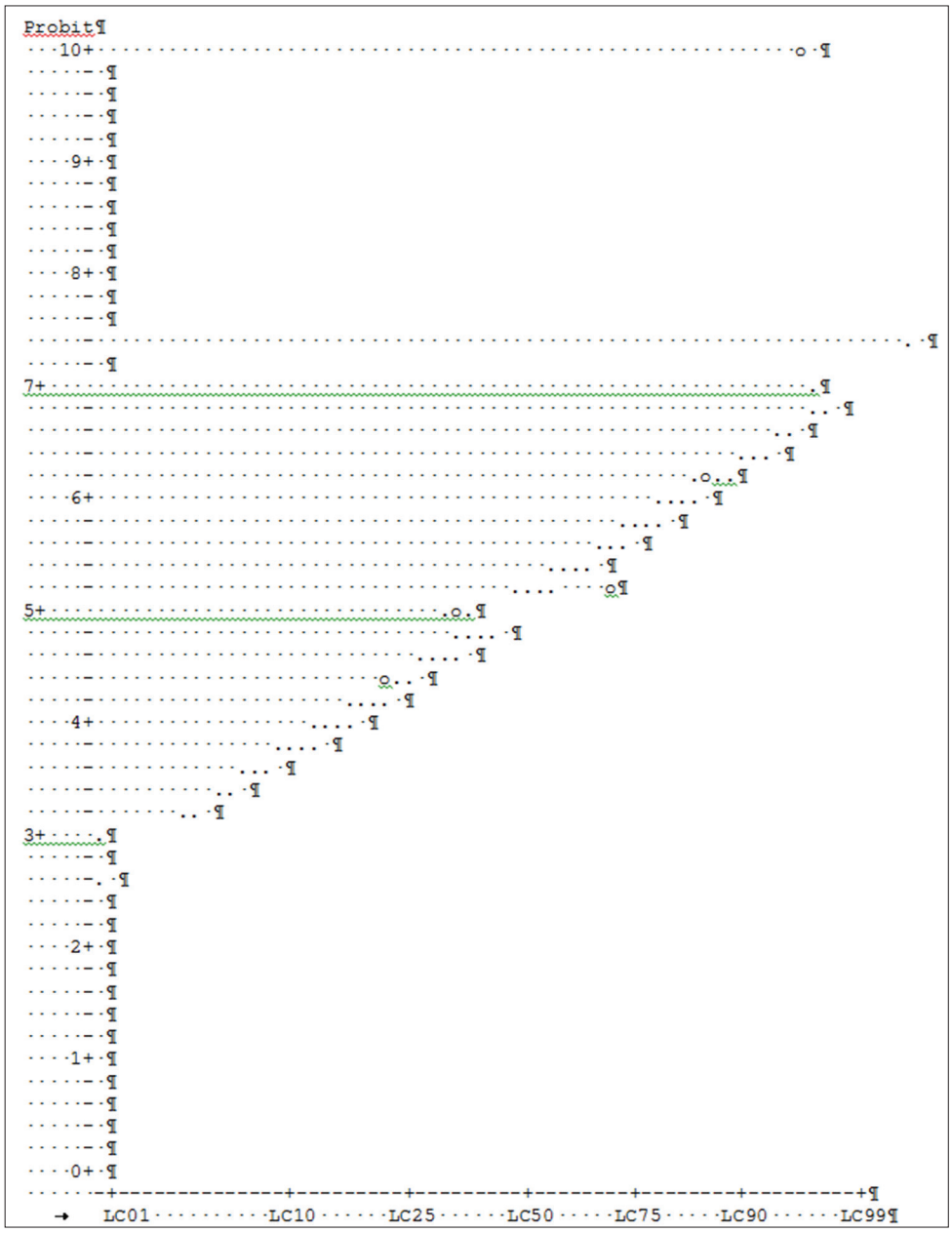

Fig. 1: Plot of adjusted probits and predicted regression line

The 96 hrs LC_{50} of the aqueous garlic extract found in the present study for the bath immersion treatment of *C. carpio* was 253.19 mg/L, and it is based on the Finney's probit analysis method; as the OECD guidelines 220 [37] advocates that this should be the method normally applied to determine LC_{50}. These findings agreed with those of Gholipour-Kanani *et al.* [38], who reported the use of 0.1 g/L garlic extract as a bath treatment against *Ichthyophthirius multifilus* infection in the ornamental fish *Poecilia latipinna*. Furthermore, Fridman *et al.* [6] have also reported that bathing of *Gyrodactylus turnbulli* infected guppies (*P. reticulata*) in 7.5 and 12.5 ml/L aqueous garlic extract significantly reduced the infection prevalence in the fish. There are also reports of other herbal extracts fairly used in fish with similar LC_{50} and also considered safe; with the examples being the 24 hrs LC_{50} of the aqueous neem (*Azadirachta indica*) leaf extract for juveniles of the neotropical fish *Prochilodus lineatus* estimated as 4.8 g/L [39], and the aqueous extract of *Terminalia catappa* tested in *Phalloceros caudimaculatus* which presented an LC_{50} of 208.52 mg/L [36]. Similarly, the LC_{50} of the *U. tomentosa* extract on *H. eques* after 48 hrs of exposure was found to be 1816 mg/L [33].

Considering the potential harm of veterinary drug treatments on the environment and human health, and in some cases their limited efficacy, there has been an increasing necessity in disease management to explore harmless, preventive, and lasting methods [16]. It is on that consideration that there has been an increase in the use of traditional, plant-based medicines in the control and treatment of diseases in fishes. Further, it is noteworthy that the widespread and long-standing use of garlic as a food ingredient for humans also suggests that garlic is non-toxic to man [6].

CONCLUSION

From the current study, it can be concluded that the aqueous garlic extract has a low toxicity on *C. carpio* and therefore can be safely used in this species for medical and prophylactic purposes. The acute toxicity data obtained from the present study thus provide a baseline information on the dosage for the aqueous garlic extract to be used as a potential management tool in aquaculture; although further investigations are required to look into the chronic effects, if any, on fish growth, survival rate, and reproduction [38].

ACKNOWLEDGMENTS

The authors express their gratitude to the Principal, Lady Keane College, Shillong for the kind permission and support enabling this research work to be carried out; the Department of Biotechnology, Government of India, New Delhi under its Institutional Biotech Hub scheme for partially funding this research work; and Ananya Barman for some of the resources provided.

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