

Evaluation on Toxicity Level of *Terminalia catappa* Leaves Extract on Selected Cyprinids under Different Bath Concentrations

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ABSTRACT

This study aimed to determine the lethal concentration (LC₅₀) of *Terminalia catappa* leaves extract on three cyprinid species; carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tiger barb (*Puntigrus tetrazona*) through the acute toxicity test. The leaves of *T. catappa* were extracted with methanol and prepared in various immersion concentrations (40, 80, 100, 150, 200, 250, 300, and 350 mg/L). These extracts were immersed in the aquarium and left for 24 h before performing the acute toxicity test. The water quality was also analyzed before and after adding the extract immersions into the aquarium. The acute toxicity test conducted for 96 h with 10 fishes of each cyprinid species (4.0-6.0 cm length) in 30 L water capacity aquarium. The mortality of each cyprinid species was recorded at 24 h time interval and LC₅₀ of the extracts throughout 96 hours was determined through the probit analysis application. Specifically, the LC₅₀ of *T. catappa* leaves extract were 349.89, 338.65 and 318.48 mg/L exhibited for carp, goldfish and tiger barb, respectively. A high concentration range of any plant-based extract has the potential to become toxic to particular fishes. Thus, it is an effort from this study to identify the safety margin of *T. catappa* leaves extract before its therapeutic values can be further manipulated and elucidated in aquaculture research.

Keywords: Leaves extract, lethal concentration, mortality, ornamental fish

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INTRODUCTION

The ornamental fish industry in Malaysia is expanding due to the increasing number of local fish exporters, even though there are still concerns over the economic viability and sustainability of this industry (Department

of Fisheries [DOF], 2016). Cyprinidae considered among the popular fish families in Malaysia for the ornamental and food fish industries. Cyprinids were on the highest rank, which contributed about RM 103 million from a total of RM 341 million in ornamental fish production in 2015 (DOF, 2015). Among the cyprinid species, carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tiger barb (*Puntigrus tetrazona*) are having a relatively high market demand due to its beautiful pattern and colors.

However, significant losses in many aquaculture industries from the uncontrollable disease outbreaks that emerge from various causes seemed to be unavoidable (Citarasu, 2012; Rodger, 2016). Progress in combating the diseases currently is time-consuming and has become slower through conventional protocols, since chemical drugs resistance problems are dramatically aroused (Liu et al., 2017; Song et al., 2015). To some extent, the over-dosage of antibiotics or antimicrobial treatments can spoil the captivating features and color of ornamental fishes, which ultimately affects its sale and market values (Marimuthu et al., 2012). As for issues related to the future of the ornamental fish industry, there are tremendous efforts and countless studies presently in search of alternative sources, especially from indigenous or herb plants that are convenient and harmless for disease treatments.

Terminalia catappa or locally known as 'ketapang' is a multi-purpose tree since almost all of its parts can utilize for human and animal benefits (Kadam et al., 2011). The

dried leaves are said to have miraculous or therapeutic effects on the infected or injured fishes, according to locals in Southeast Asia (Chansue & Assawawongkasem, 2008). Therefore, it is common to see the leaves submerging in the culture tanks of fish breeders or even home aquariums as protection from diseases for their cultured fishes. *Terminalia catappa* leaves typically contain various phytoconstituents such as tannins, isovitexin, flavonoids and triterpenoids (Citarasu, 2010), which have proven efficient as fish anti-pathogenic through many *in vitro* tests (Allyn et al., 2018; Fakoya et al., 2019; Purwani et al., 2015; Walczak et al., 2017). The *in vivo* studies through bath treatment of these leaves on the infected fishes are still scarce and selective in certain species of ornamental fishes particularly on Siamese fighting fish (*Betta splendens*) and guppy fish (*Poecilia reticulata*) (Chansue & Assawawongkasem, 2008; Nugroho et al., 2017; Purivirojkul, 2012). Many researchers are likely more interested in the *in vitro* test compared to *in vivo* tests, as the preparations seem complicated with its toxicology and safety evaluations on live animals (Akinsanya et al., 2016; Manaharan et al., 2014). Although there are increasing numbers in plant remedy studies, their scientific works on its safety, toxicity and adverse effects for the specific host or target are not always discovered together at the same time (Kasthuri & Ramesh, 2018; Saad et al., 2006).

With the main focus on the immersion concentrations of *T. catappa* leaves extract, the present study aims to determine its toxicity level on the three selected cyprinid species, which are carp, goldfish and tiger barb. Information from this toxicity study would serve as an essential baseline for further studies in establishing *T. catappa* leaves extract as an alternative source of therapeutics against diseases in ornamental fishes.

MATERIALS AND METHODS

Preparation of *Terminalia catappa* Extract

The green leaves of wild-grown *T. catappa* were collected and dried under the sunlight for about one week with another 48 h drying with oven (Protech®-FSD 380) at 40°C. These dried leaves were pulverized to fine powders with a commercial blender (Butterfly® B-592) and freeze dried at -45°C (Labconco-7750030) as preparations for methanolic extraction. The extractions were performed for about 48 h using 100 g of *T. catappa* leaves powder with 1000 mL methanol according to the method by Yi et al. (2012). The excessive methanol was then evaporated using rotary evaporator (Eyela-N1100) and the obtained crude extract were kept in the freezer (-20°C) and used as a test solution.

Preparation of Fish Culture

The selected cyprinid species of carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tiger barb (*Puntigrus*

tetrazona) purchased from the local breeders; considerably similar in size (4.0-6.0 cm length). Prior to experiment, all fishes acclimatized under the laboratory conditions for seven days before conducting the acute toxicity test. The dissolved oxygen levels maintained within the standard concentration range for freshwater fishes between 3.0 to 7.0 mg/L (DOF, 2016) and the fishes were daily fed once with commercial fish pellet.

Acute Toxicity Test

Eight different concentrations of *Terminalia catappa* leave extract (40, 80, 100, 150, 200, 250, 300, and 350 mg/L), each with three replicates along with one control were set up in this test. The test conducted according to the method of Ekanem et al. (2004) within 96 h. The extracts initially immersed for 24 h before adding the 10 fishes of each cyprinid species into the 30-L tank aquarium. The water was continuously aerated without any change and fed not provided throughout the test. Any dead fish found under these circumstances during the observation at every 24 h was isolated from the aquarium. The total of death fishes throughout 96 h in the respective extract concentrations was finally recorded for determining the LC₅₀ or lethal concentration of the extracts.

Water Quality Analysis

The quality of aquarium water with non-immersions and immersions of leaves extract was analyzed before performing the acute toxicity test. The pH, temperature,

dissolved oxygen and total ammonia-nitrogen of the water were measured using water quality probe (YSI Pro Plus). The final concentration of total ammonia-nitrogen was further determined using a Nessler-2458200 reagent set.

Statistical Analysis

Statistical analysis using one-way analysis of variance (ANOVA) was applied to the results of water quality analysis. The significant differences at 95% confidence level between the data samples distinguished using Duncan's multiple range test (Duncan-MRT). The lethal concentration or LC₅₀ of *T. catappa* leaves extract on the tested cyprinid species was determined using probit analysis.

RESULTS

Acute Toxicity Test

No mortality was observed within the 96 h exposure for all cyprinid species in the concentrations ranging from 20 to 200 mg/L (Tables 1, 2, and 3). However, the mortality significantly increased when the tested concentrations distinctly increased from 250 to 350 mg/L. The highest bath concentration of 350 mg/L also demonstrated almost to 100% mortality in all cyprinid species, especially tiger barb.

Probit analysis was applied to determine the relative LC₅₀ or lethal concentration of *T. catappa* that caused death in at least 50% from the tested cyprinid species in a specified period of 96 h. Through this analysis, different LC₅₀ of *T. catappa* leaves

Table 1

Mortality rate (%) of carp (*n*=10) within 96 h bath immersion of *Terminalia catappa* leaves extract at different concentrations

Time (h)	Concentrations of extract (mg/L)								
	Control	40	80	100	150	200	250	300	350
24	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	10
72	0	0	0	0	0	0	0	10	20
96	0	0	0	0	0	0	20	20	50
Total (%)	0	0	0	0	0	0	20	30	80

Table 2

Mortality rate (%) of goldfish (*n*=10) within 96 h bath immersion of *Terminalia catappa* leaves extract at different concentrations

Time (h)	Concentrations of extract (mg/L)								
	Control	40	80	100	150	200	250	300	350
24	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	10
72	0	0	0	0	0	0	0	20	50
96	0	0	0	0	0	0	20	20	30
Total (%)	0	0	0	0	0	0	20	40	90

extracts were obtained on carp, goldfish and 338.65 mg/L (Figure 2), and 318.48 mg/L tiger barb with 349.89 mg/L (Figure 1), (Figure 3), respectively.

Table 3
Mortality rate (%) of tiger barb (n=10) within 96 h bath immersion of *Terminalia catappa* leaves extract at different concentrations

Time (h)	Concentrations of extract (mg/L)								
	Control	40	80	100	150	200	250	300	350
24	0	0	0	0	0	0	0	0	20
48	0	0	0	0	0	0	0	10	40
72	0	0	0	0	0	0	10	10	40
96	0	0	0	0	0	0	20	40	0
Total (%)	0	0	0	0	0	0	30	60	100

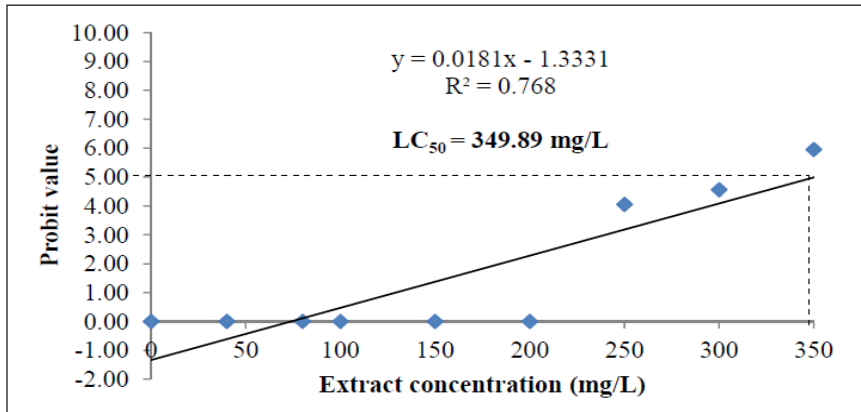


Figure 1. Probit analysis of determining the LC_{50} of *Terminalia catappa* leaves extract on carp (*Cyprinus carpio*) based on total mortality within 96 h

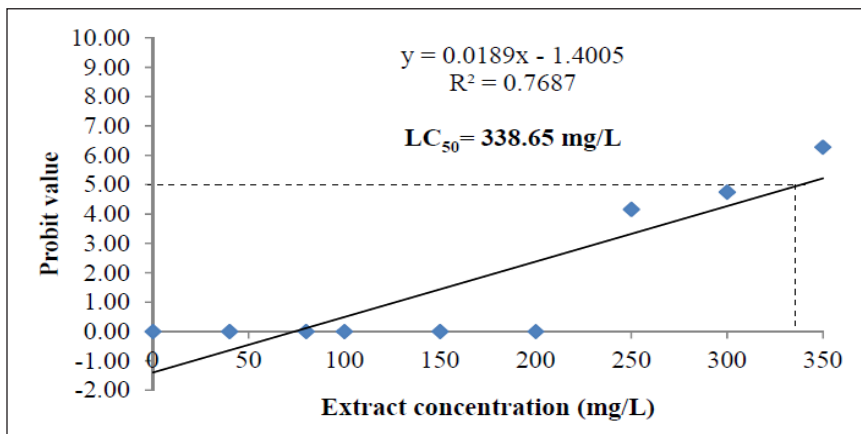


Figure 2. Probit analysis of determining the LC_{50} of *Terminalia catappa* leaves extract on goldfish (*Carassius auratus*) based on total mortality within 96 h

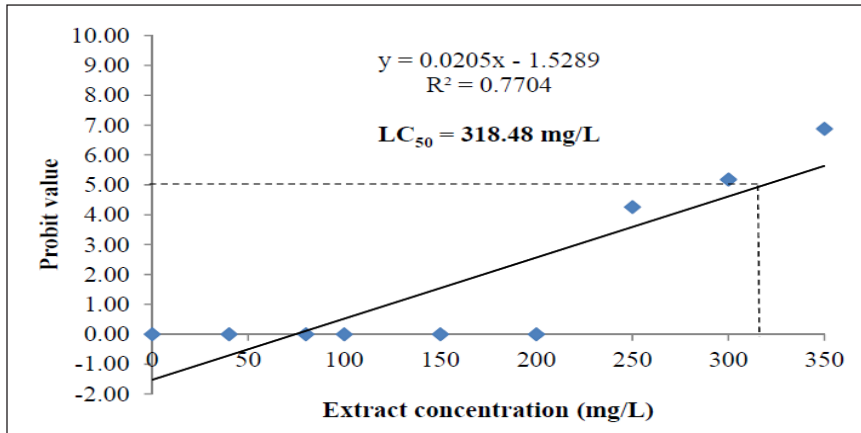


Figure 3. Probit analysis of determining the LC₅₀ of *Terminalia catappa* leaves extract on tiger barb (*Puntigrus tetrazona*) based on total mortality within 96 h

Water Quality Analysis

The mean values for water quality analysis showed a paralleled increment between the extract concentrations and total ammonia-nitrogen concentrations but conversely different between the pH (Table 4).

DISCUSSION

The acute toxicity test of *T. catappa* leaves extract in this study revealed at a

specific concentration range of its bath immersion could cause toxic and mortality on selected cyprinid species. Our findings were in agreement with Chansue and Assawawongkasem (2008)’s as well as Stratev et al. (2018)’s studies, which stated that any natural or herbal plant source could either be beneficial or detrimental to any fish since the level of its toxicity mainly depended on the applied extract

Table 4
Water quality parameters of different concentrations of *Terminalia catappa* leaves extract for toxicology assessments on tested cyprinid species

Concentration of extract (mg/L)	pH	Total ammonia-nitrogen (mg/L)
Control (no extract)	6.92 ± 0.05 ^a	0.43 ± 0.06 ^a
40	6.75 ± 0.04 ^b	0.82 ± 0.06 ^b
80	6.70 ± 0.02 ^{bc}	1.15 ± 0.06 ^c
100	6.64 ± 0.04 ^{cd}	1.45 ± 0.04 ^d
150	6.61 ± 0.04 ^{de}	1.91 ± 0.03 ^e
200	6.54 ± 0.04 ^{ef}	2.22 ± 0.04 ^f
250	6.51 ± 0.03 ^f	2.82 ± 0.04 ^g
300	6.42 ± 0.01 ^g	>3.5 ^h
350	6.39 ± 0.03 ^g	>3.5 ^h

Note. Data (mean) showed in two replicates with standard deviation. Values with the different letter (^{a-h}) in each column are significantly different at p < 0.05 based on Duncan-MRT

concentrations and targeted fish physical and species. Chansue and Assawawongkasem (2008) discovered that guppy (*Poecilia reticulata*) and Siamese fighting (*Betta splendens*) fishes (approximately 2.0 cm in length) had different toxicity effects by water extract of *T. catappa* leaves. Even if they are averagely similar in size, the guppy fish was found to be more sensitive than Siamese fighting fish with the LC₅₀ for 96 h of 5.6 and 7.0 mg/L, respectively. Another study by Purivirojkul (2012) showed high LC₅₀ (1,765.7 mg/L) of the *T. catappa* leaves extract on similar fish species utilized in Chansue and Assawawongkasem (2008)'s study; Siamese fighting fish but it is slightly bigger (about 4.0 cm in length) than the previous one. Furthermore, the concentrated plant extracts may also contribute to a severe cause to the non-target aquatic species in certain circumstances (Singh & Singh, 2002; Yunus et al., 2019). Therefore, a toxicological assessment of the plant extracts is indeed essential to conduct meticulously prior to usage on the targeted fish species or before other manipulation of the extract can further investigate.

The changes in pH value were observed where they were gradually reduced to acidic from the low to high concentrations of leaves extract in this study. These conditions were expected as *T. catappa* had the potential to lower the pH of water (Lee et al., 2016). However, *T. catappa* leaves extract at a low concentration ranging from 2.0 to 5.0 mg/L had proven to be non-effective on the acidity or alkalinity of the water, according to Bryan (2016). It showed acidic (pH 6.5-

6.0) with 6.0 to 10.0 mg/L concentrations from the leaves extracted for three days using water in the study of Chansue and Assawawongkasem (2008). Most of the cultured fishes were well-tolerated with the pH ranges from 6.2 to 7.8 (Chansue, 2007; Chitmanat et al., 2005). Their growth and mortality might be affected and resulted from the rapid change of pH over 0.2 (Chansue & Assawawongkasem, 2008). Stone et al. (2013) also stated almost similar findings with Chansue and Assawawongkasem (2008), where the acceptable pH range for most fish species was within 6.5 to 9.0 and chronic pH level (below 6.5) may reduce the fish reproduction. Even our concentrations of the leaves extract varied, the acidic pH ranges and its effects on the tested fishes showed an agreement with the previous studies. No mortality was recorded in all tested cyprinid species between 40 and 200 mg/L of leaves extracts with acidic conditions within a pH of 6.75 to 6.54. The mortality of cyprinid species was only exhibited between 250 and 350 mg/L concentrations with lower pH than 6.54.

In general, many factors can contribute to the pH changes in the fish culture environment or condition. Ikhwanuddin et al. (2014) stated that the decreased pH or acidic conditions in any plant-based bath immersion could be reflected from the accumulation of its organic acids especially tannins. Tannin, a polyphenolic compound commonly found in most herbal plants may be employed as a source of alternative treatments in its adequate concentration as antibacterial, antiparasitic, antiviral and

antifungal for many fish species (Azrul et al., 2014; Chansue & Assawawongkasem, 2008; Chitmanat et al., 2005; Yunus et al., 2019). However, the excessive and high level of tannins in the water and feed formulation showed adverse effects on herbivorous and omnivorous fishes (Azrul et al., 2014; Mandal & Gosh, 2010). Chansue and Assawawongkasem (2008) found that heavy solid suspension adhered to the gills of guppy and Siamese fighting fishes through necropsy tests upon the completion of *T. catappa* leaves extract toxicity test. These findings could be evidence of irritation since the adhered gills were blocked for oxygen by a high concentration of tannins contained in the applied *T. catappa* leaves extracts. In the study of Borisutpeth et al. (2001), tannic acid at 97.5 mg/mL caused hyperplasia in epithelial cells of gill filaments, aneurysm of gill lamellae and disarray in tilapia; which ultimately died without histopathological changes in other organs within 96 h. Accumulation of significant level of tannin also found in the different fish species of Indian major and exotic carps and its tissues (liver and kidney) because of the tannin-like compounds presented in their feeding (Mandal & Gosh, 2010). Since no histopathological tests performed to support our findings, we stipulated that tannins at their high concentration levels and other active compounds (flavonoids, saponin, calcium oxalate and glycosides) contained in the *T. catappa* leaves (Tercas et al., 2017) have the potential to decrease the pH and ultimately caused mortality in the tested cyprinid species.

Total ammonia-nitrogen (TAN) is also another significant contributor to fish mortality other than tannins. Ammonia ionizes and deionizes the aquaculture system into NH_4^+ and NH_3 , respectively, which can be toxic depending on the levels present and fish species tolerance (Roberts & Palmeiro, 2008). Generally, any decomposing organic residue from the plant and fish waste has a great potential to raise the ammonia and pH levels in the water as protein break downs (Ip & Chew, 2010; Yavuzcan et al., 2017). Malaysia has set specific standards for acceptable and safety levels of TAN (ionized and unionized) for freshwater and marine fishes at 0.3 mg/L (DOF, 2016; White et al., 2008). At a low concentration of deionized ammonia of 0.05 mg/L, harmful effects such as poor growth rate, reduced fertility, increased stress, and susceptibility to disease could result in fish (da Silva et al., 2013). Meanwhile, at a high concentration that exceeds 2.0 mg/L, gills, and tissues can be damaged and ultimately causes mortality and death to the fish (Ip & Chew, 2010). TAN concentration in the present study found slightly higher in the control but extremely elevated (>3.5 mg/L) when the concentrations of leaves extract concurrently increased (Table 3). These evidences have enough to demonstrate that insignificant TAN concentration responsible to cause toxics to all tested cyprinid species in this study. However, other factors that influence the vulnerability of targeted fishes on TAN should consider as well. The toxicity level of TAN according to the standard is also dependent on the fish's

biological traits, sensitivity and adaptation levels to the affected environmental system (Wang et al., 2016). Remarkably, saltwater fish species found to be more sensitive to ammonia toxicity effects as compared to freshwater fish species according to Roberts and Palmeiro (2008). Therefore, we found that the tolerable capacity of our tested fishes varied towards the changes in their living environment even if they are from the same family. Goldfish managed to show higher tolerance than carp and barb in acidic pH condition and TAN concentration caused by the leaves extract immersion of *T. catappa*. Hence, overall results from this study can contribute significant knowledge to the aquaculture and local communities on the safety margin of these leaves to be utilized for their cultured fishes and provide promising insight into other potential use of *T. catappa* leaves extracts in fish ornamental industry.

CONCLUSION

The present study showed different effects of cyprinid species in various bath immersions of *T. catappa* leaves extract. The most tolerable threshold or safety concentration boundaries of the *T. catappa* leaves extract for all tested cyprinids were obtained up to 200 mg/L, where no mortality recorded among them. All tested cyprinids shared an almost similar LC₅₀ between 300 and 350 mg/L. Further study based on these findings may be necessary to investigate the effectiveness of these leaves extract in improving the cyprinids' health and growth in a long term treatment.

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