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The Effects of *Terminalia catappa* L. Leaves Extract on the Water Quality Properties, Survival and Blood Profile of Ornamental fish (*Betta* sp) Cultured

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Abstract

This research aimed to determine the phytochemicals content of *Terminalia catappa* leaves extract (TCL) and its effects on the survival and blood profiles of ornamental fish (*Betta* sp) Ninety fish were randomly assigned into six triplicates groups and reared in various concentration of TCL: 0 (control), 125, 250, 375, 500, 625 ppm for 30 days. Temperature, Dissolve oxygen (DO), and pH were monitored during the trial. After 30 days, survival, Red Blood Cells (RBC), White Blood Cells (WBC), haemoglobin (Hb), lymphocyte, and total protein serum (TPS) were analyzed. Based on the phytochemicals test, saponin, triterpenoid, quinon, phenolic, tannin, and flavonoid were detected on the TCL. Temperature and DO were not affected by any concentration of TCL. The lowest pH (5.05) was found in fish medium immersed with 625 ppm of TCL. Adding TCL above 375 ppm resulted in significantly higher survival, RBC, and Hb. The highest WBC was found in fish immersed with 625 ppm whereas the lowest lymphocyte was found in fish immersed with 375 of TCL. However, immersing any various concentration of TCL did not affect on the TPS. In summary, immersing TCL above 375 ppm is beneficial to enhance survival, RBC, WBC, and Hb of *Betta* sp.

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INTRODUCTION

Terminalia species are widely distributed both in tropical and sub-tropical regions, including Asian (Hyttel et al., 2009; Hyttel et al., 2010). In Indonesia, *Terminalia catappa* L. is an attractive, long-lived tree well suited to ornamental and amenity plantings. The water extract of *Terminalia catappa* leaves (TCL) has also been known as a folk medicine for antipyretic, haemostatic, hepatitis and liver-related diseases purposes in Philippines, Malaysia and Indonesia (Meena & Raja, 2006; Vučurović & Razmovski, 2012).

Besides as the folk medicine, TCL has also been used in fish culture and breeder in various ways. Chitmanat et al. (2003) proved that TCL can be applied in Tilapia (*Oreochromis niloticus*) culture to protect the fish against the pathogen. The TCL sometimes referred as a miracle leaf by fish breeders across the globe, claiming its usefulness in creating spawns and citing wondrous healing properties. According to Pandey (2013), TCL has a potential as herbal biomedicine to improve non-specific defense mechanism of fish and elevate the specific immune response. The TCL is also said to be responsible for, lowering pH, and treating water hardness (kH). Although the claims are tremendous and vast, the TCL has little scientific research done on all of the claims of its beneficial properties, especially on hematological profile of fish.

The immune status of fish is related to haematology condition which is importance in fish culture because of its value in monitoring the health status of fish (Chandel et al., 2009). Haematological condition such as total erythrocyte count (RBC), white blood cells (WBC) count, haemoglobin count (Hb) and total protein in the serum of fish is an effective tool that can be used to evaluate physiological and pathological changes in fish (Inama et al., 1993). Moreover, WBC has been regularly used as indicators of health status in fish because WBC is key components of innate immune defense and involved in regulation of immunological function in fish including ornamental fish (*Betta* sp) (Zhou et al., 2010; Ekman et al., 2013).

Ornamental fish, *Betta sp*, belongs to the Labyrinth fish family (Belonitiidae) and known as a colorful species, especially in males (Ekman *et al.*, 2013). As an important cultured species in Indonesia, *Betta sp* is recognized as a valuable fish commodity, increasing market demand for this fish has led to a significant boost in research to improve survival and health performance of the fish. However, the information regarding the effects of TCL on the medium water quality, survival and blood profile of *Betta* sp is limited. Thus, the current study was designed to evaluate, water quality in the medium (Temperature, Dissolve oxygen, and pH), survival rate, RBC, WBC, total Hb, the percentage of lymphocyte, and total protein serum in the blood of *Betta sp* exposed to different concentration of TCL. Also, the phytochemical compounds in the extract which probably responsible for blood profiles properties were also identified.

The outcome of this research is beneficial to determine the benefit of the phytochemicals in TCL to improve the physiological condition of *Betta* sp. Further, the optimum concentration of TCL immersion which was obtained in this research can be used to enhance the health of *Betta* sp as indicated by survival and blood profile.

METHODS

Plant Material

Terminalia catappa's dried cut brown leaves were collected on the campus of Mulawarman University, Barong Tongkok, East Kalimantan, Indonesia. In purpose of eliminating the extraneous matter, thecollected *T. catappa's* leaves were washed with deionized water and immediately dried in an oven at 40°C for 12 h. *T. catappa's* powder (8 opening cm^{-1} passed) was obtained using a mill. The powder was extracted with ethanol 95% for three days (100 g L^{-1}). After filtration, the extract was evaporated by using rotary evaporator and stored at 4^0C until used as a crude extract.

Preliminary Phytochemical Tests

The preliminary phytochemical tests such as alkaloid, saponin, steroid, triterpenoid, quinon, phenolic, tannin, and flavonoid were performed to detect the presence of possible phytochemicals in the extract of *T. catappa* leaves, they are: (1) Test for alkaloid-Dragendorff Test: 2 mL of the filtrate was added by 1 mL of Dragendorff reagent along the side of the test tube. Formation of orange or orange reddish brown precipitate indicates a positive test. (2) Test for saponin, 1 mL of extract + 5 mL distilled water was shaken vigorously, the appearance of stable froth (1-3 height) for 15 minutes indicates the presence of saponin. (3) Test for steroids and (4) triterpenoid (Liebermann-Burchard), 2 mL extract + 1 mL chloroform + few drops of acetic anhydride + conc. sulphuric acid added along the side of the test tube), the appearance of blue or green colour indicated the presence of steroids, and appearance of red, brown colour indicates the presence of triterpenoid. (5) Test for Quinon, 1 mL of extract + 1 mL of conc. sulfic acid. A red coloration indicated the presence of quinon. (6) Test for phenols and tannins: crude extract was mixed with 2 mL of 2% solution of FeCl3. A blue-green or black coloration indicated the presence of phenols and tannins. (7) Test for flavonoids (2 mL extract + conc. hydrochloric acid + magnesium ribbon), the appearance of pink-red colour indicated the presence of flavonoids.

Animals and Experimental Setup

Ninety Siamese fighting fish (male with average initial weight 0.62 g; Crown tail), 2-month-old, were purchased from Local breeding Farm, Samarinda East Kalimantan and acclimated at Animal Physiology, Development, and Molecular Laboratory, Mulawarman University, East Kalimantan for a week. Fish were then randomly distributed into six triplicate groups of five Siamese fighting fish each group. Each group of Siamese fighting fish was then placed in a glass tank (0.5 L capacity, 0.4 L of fresh water in each tank. The study was conducted for 30 days, fish in each group of treatment was added with a various concentration of TCL *viz* 125, 250, 375, 500, 625 ppm as immersion. Temperature, pH, and Dissolve Oxygen (DO) were measured every second-day using a routine thermometer, pH meter, and TOA-dkk pH HM-7, TOA instrument, Japan. The fish in each glass tank was fed with frozen bloodworm at a rate of 1% of body weight every day. Previous experiments determined this feeding rate. Uneaten food and faeces were siphoned out before renewing the water. The water volume was renewed every second day using each treatment concentration and maintained to 0.4 L of water in every glass tank.

Sampling and Analytical Procedure

The survival rate of fish in each group was recorded every 10 days. At the day 30, blood samples were taken by caudal venipuncture after anaesthetizing the fish with MS-222 (200 mg L^{-1}). Total RBC (10^6 per mm^3) and WBC (10^3 per mm^3) were determined manually with the improved Neubauer counting chamber. Haemoglobin was determined according to the cyanmethemoglobin procedure (Blaxhall & Rao, 1973) and expressed in g dL^{-1}. Lymphocyte (10^3 μL^{-1}) was determined by using Auto Hematology Analyser Mindray BC2800, Mindray® Shenzhen, China. In order to measure serum protein in the blood, 0.1 mL of fish blood was withdrawn, mixed with 0.1 mL of EDTA and dissolved in 0.2 mL of NaCL 0.9%. The mixture was centrifuged 1000 rpm for 15 minutes. The supernatant was used for serum protein determination by using Biolis 24i, Tokyo Boeki Machinery Ltd. Japan.

Statistical Analysis

Results are expressed as means ± standard error (SE) and data were analyzed using SPSS version 22 (SPSS, Inc., USA). The data of Survival was transformed to arcsine, water quality, RBC, WBC, total Hb, the percentage of lymphocyte, and total protein serum on the day 30 of the trial were subjected to one-way ANOVA, followed by Duncan post hoc test to evaluate significant differences among the group of treatments. All significant tests were at $P<0.05$ levels.

RESULTS AND DISCUSSION

Water Quality

Fish have a close relationship with their surrounding aquatic environment. Most aquarium fish such as *Betta* sp lives in a closed system which is mean that water needs to be manually removed and added to be renewed. The TCL has traditionally been used by *Betta* sp breeders in South East Asia, including Indonesia to mimic the natural *Betta* sp habitat. The TCL contains many hydrolyzable tannins, namely terflavins A, terflavins B, tergallagin, tercatin, punicalin, punicalagin, chebulagic acid, geraniin, granatin B, and corilagin (Tanaka et al., 1986; Chen & Li, 2006).

Based on the present results, temperature and DO were not affected by any concentration of TCL immersion whereas immersion 625 ppm of TCL showed significantly lowest pH (5.05 ± 0.10) in the fish medium (Table 1). The decline in pH could be due to the tannin content in the TCL. According to Zhao *et al.* (1999), tannins have potential commercial applications in aquaculture. However, tannin may reduce water pH (Miksen & Media, 2016).

Phytochemical Content

Numbers of plant extract which have active ingredients and various biological activities have been reported as supplementation in aquaculture field (Citarasu, 2010; Madhuri et al., 2013; Chakraborty et al., 2014; Sivasankar et al., 2015; Syahidah et al., 2015). However, data regarding the phytochemical of *T. catappa* and its effect on the fish, especially *Betta sp* is scarce. Practically, the screening test of the phytochemical compound in *T. catappa* is important to be done to find out the benefit of plant bioactive agents in fish. The use of ethanol as an extractor is due to its capability to bind phytochemical compounds

Table 1. Water quality of *Betta* sp-immersed medium with a various concentration of *Terminalia catappa* leaves extract.

Variables	Control	125 ppm	250 ppm	375 ppm	500 ppm	625 ppm
Temperature	25.57 ± 0.030	25.58± 0.016	25.57 ± 0.054	25.73 ± 0.223	25.62 ± 0.014	25.62 ± 0.014
DO	3.35 ± 0.193	3.48 ± 0.073	3.37 ± 0.073	3.05 ± 0.220	3.43 ± 0.166	3.43 ± 0.166
pH	6.14 ± 0.008[a]	6.125±0.014[a]	6.175 ± .014[b]	6.21 ± 0.464[b]	6.25 ± 0.166[b]	5.05 ± 0.100[c]

Data are given as mean values (Mean ± SE). Different superscript (a, b, c) in the same row indicates significantly different mean values at *P<0.05*. *Terminalia catappa* leaves extract was added as an immersion. Temperature = (^{0}C); DO = (mg mL^{-1})

such as tannin, polyphenol, flavonoid, terpenoids, and saponin (Cowan, 1999; Ayini et al., 2014). The current study indicated the presence of saponin, triterpenoid, quinon, phenolic, tannins, and flavonoid in the TCL (Table 2).

Table 2. Phytochemical screening test of *Terminalia catappa* leaves extract

Phytochemicals	Results
Alkaloid	-
Saponin	+
Steroid	-
Triterpenoid	+
Quinon	+
Phenolic	+
Tanin	+
Flavonoid	+

(+) Present; (-) Absent

Saponin is considered as anti-nutritional in field aquaculture. However, previous experiments stated that saponin had several beneficial effects on some fish such as common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) (Stadlander, 2012). It is also revealed that low level of saponin was improved growth rate, feed conversion efficiency, protein utilization and reduced oxygen consumption. Also, saponin had various effects on the physiological status such us, increasing the number of RBC, hemoglobin, hematocrit, oxygen uptake and binding capacity in Perch (*Anabas testudineus*) (Roy & Munshi, 1989).

Besides saponin, another group of phytochemicals, such astriterpenoid, quinon, phenolic are also a common plant active compound that can be found in TCL and has been reported to promote various activities like anti-stress, growth promotion, appetite stimulation, tonic, and immune booster (Citarasu, 2010; Chakraborty et al., 2012; Chakraborty *et al.*, 2014). Meanwhile, tannins and flavonoid which were a presence in TCL are the main group of plant phenolic compound that act as primary antioxidants or free radical scavengers. According to Chansue and Assawawongkasem (2008), tannin from *T. catappa* extract with water has the ability as an antibacterial substance for ornamental fish. The tannin could inhibit the growth of bacteria in intestinal of fish by binding iron and form a chelate. On the one hand, flavonoid is one of the most widespread groups of phytochemicals in the plant that was shown to improve reproduction of Japanese Medaka (*Oryzias latipes*) (Kiparissis, 2001). It acts as antimicrobial properties on fish pathogen (Rattanachaikunsopon & Phumkhachorn, 2007), and as a growth promoter on juvenile *Pargus major* (Ji et al., 2007), *Carassius auratus* (Ahilan. B et al., 2010), *Catla catla* (Kaleeswaran et al., 2011). Thus, the presence of secondary metabolites in TCL might play a role in the survival and blood profile of *Betta* sp.

Survival Rate

According to Chakraborty & Hancz (2011), phytochemicals such as alkaloids, flavonoids, pigments, phenolics, and terpenoids are secondary metabolites in plants. The phytochemicals contained in the plant may enhance the innate immune system that may be of immense use in fish culture. Further, the phytochemical may improve the survival rate of the fish. Some previous studies have been conducted to evaluate the use of phytochemicals from various parts of plants extract to enhance the survival rate of the Common Carp (*Cyprinus carpio*); marine ornamental fish (Balachandran & Tissera, 2013), and tilapia (*Oreochromis niloticus*) (Akinwande et al., 2011).

The present study indicated that *Betta* sp added with the immersion of TCL above 375 ppm showed significantly higher (*P<0.05*) survival rate. The highest survival rate (Fig. 1) was found in *Betta* sp added 625 ppm of TPC. This finding was in line with previous research that there was no significant evidence on the mortality of *Betta* sp after exposure of phytochemicals (Clotfelter & Rodriguez, 2006). Furthermore, Ashraf and Bengtson (2007) revealed that the

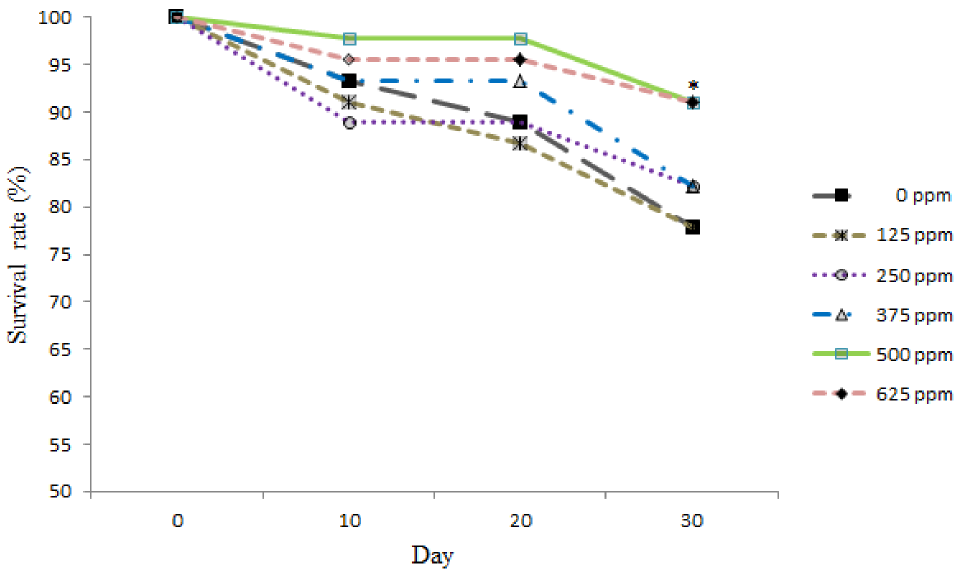

Figure 1. Survival rate (%) of ornamental fish (*Betta* sp) after various immersion concentration (ppm) of *Terminalia catappa* extract for 30 days. (*) The highest survival rate (625 ppm of TCL)

presence of tannin in feeding had positive effects on the survival of larval striped bass (*Morone saxatilis*). However, some previous studies stated that tannin which presents dominantly in plant had negative effects in *Channa straitus* and *Cyprinus carpio* (Viswaranjan et al., 1988), the use of tannin can absorb harmful chemicals, give soothing and suitable environment fairly benign for fish (Van–Sumere et al., 1975; Ashraf & Bengtson, 2007).

The biological effects of saponin including survival and immune system, on fish, have also been widely studied and reviewed by several scientists. Saponins are found in plants, contain either a steroid or triterpenoid aglycone to which one or more sugar chains are attached (Oda et al., 2003). Furthermore, the use of 1 and 2 mg L^{-1} saponin in white shrimp *Litopenaeus vannamei* increased the survival rate of shrimp (Su & Chen, 2008). In contrast, it was observed that pure saponin at high levels caused severe stress and mortality, following exposure to 200 mg L^{-1} (Nagesh et al., 1999).

Blood Profile

Currently, application of phytochemical on the haematological indices is common in the culture of aquatic animals, including fish. Haematological parameters are gaining increasing importance in aquaculture because of its value in assessing the health status of fish (Schütt et al., 1997; Kori-Siakpere et al., 2005; Metin et al., 2008; Karimi et al., 2013; Suely et al., 2016). Haematological parameters such as RBC, WBC, Hb, lymphocyte, and TPC are pivotal role in assessing the physiological condition of the fish. Based on the current results found that groups of fish treated with TCL above 375 had higher RBC, WBC, and Hb than any others group whereas the lowest lymphocyte resulted in fish treated with 125 ppm. However, TPC of fish was not affected by any treatments. The RBC, WBC, Hb, lymphocyte are used as an indicator of haematological status in fish in related to innate immune defence and regulation of immunological function in the organisms (Ballarin et al., 2004).

The underlying mechanism(s) whereby TCL boosts the haematological indices of *Betta* sp is not properly understood. However, Nair, et al. (2002); Lyu and Park (2005) stated that flavonoid from plant metabolite may contribute to promoting cellular immunity by modulating Th-1 derived cytokines such as IL-2 (Interleukin 2) and INFγ (Interferon). Flavonoid can be a bio catalysator to produce leukocytes and stimulate leukocytes as nonspecific cellular immunity. Also, flavonoid has a positive effect of reducing RBC hemolysis, by protecting biological membranes of RBC from free radical which induces oxidative damage (Kitagawa et al., 1992; Asgary et al., 2005). Also, some investigators reported that the antioxidants present in the plant extract might trigger erythropoiesis and antioxidant activity (Shadu et al., 2006; Vargeshe et al., 2010; Lestari & Susanti, 2015). It seems in agreement with the present study as the TCL has flavonoids that capable as an antioxidant which may maintain the heme iron in its ferrous state and this could enhance erythropoiesis (Akah et al., 2008; Uboh et

Table 3. Blood profile of *Betta* sp added various concentrations (ppm) of *Terminalia catappa* leaves extract for 30 days

Parameter	Groups (ppm)					
	Control	125	250	375	500	625
RBC (10^6 µL^{-1})	1.846 ± 0.007^{a}	1.842 ± 0.007^{a}	1.846 ± 0.019^{a}	1.853 ± 0.027^{a}	2.014 ± 0.050^{b}	2.014 ± 0.033^{b}
WBC (10^3 µL^{-1})	4.345 ± 0.073^{a}	4.376 ± 0.071^{ab}	4.606 ± 0.265^{ab}	5.006 ± 0.313^{abc}	5.138 ± 0.306^{bc}	5.430 ± 0.227^{c}
Haemoglobin (g dL^{-1})	7.400 ± 0.048^{a}	7.520 ± 0.015^{ab}	7.749 ± 0.197^{ab}	7.748 ± 0.070^{ab}	8.414 ± 0.076^{c}	8.581 ± 0.117^{c}
Lymphocyte (10^3 µL^{-1})	65.533 ± 0.371^{a}	62.600 ± 1.205^{b}	64.000 ± 0.577^{a}	63.000 ± 0.808^{ab}	62.866 ± 0.480^{ab}	63.466 ± 0.751^{ab}
Total protein serum (mg dL^{-1})	0.492 ± 0.006^{a}	0.518 ± 0.005^{a}	0.492 ± 0.010^{a}	0.509 ± 0.003^{a}	0.500 ± 0.007^{a}	0.506 ± 0.005^{a}

Data are given as mean values (Mean ± SE). Different superscript (a, b, c) in the same row indicates significantly different mean values at $P < 0.05$. *Terminalia catappa* leaves extract was added as immersion. RBC = Red Blood Cells; WBC = White Blood Cells.

al., 2010; Shatoor, 2011).

Though saponin that is naturally occurring surface-active glycosides has been reported to have a toxic effects at high level on fish (Francis et al., 2002), Roy and Munshi (1989) revealed that there was an increasing in the erythrocyte, haemoglobin and packed cell of *Anabas testudineus* after exposure to 5 mg of saponin per liter in the water for 24 h. Nevertheless, the mechanisms of the immune-stimulating action of saponin in TCL are not clearly understood. Previous research predicted that saponin might induce the production of cytokines such as interleukin and interferon that could mediate their immunostimulant effects (Rajput et al., 2007; Helal & Melzig, 2011). Also, saponin enhances not only macrophage but also T-cell activities in finfish (Xiong et al., 2012).

CONCLUSION

Immersion above 375 ppm of TCL, containing some beneficial phytochemicals such as tannin, flavonoid, and saponin in the *Betta* sp is recommended to enhance survival and blood profile. The water quality, especially temperature and DO was not affected by immersion of TCL. However, giving high TCL concentration (625 ppm) in the fish medium reduced pH. Further research needs to be conducted to validate the effects of TCL immersion on the antioxidant enzymes activity, such as glutathione peroxide, superoxide dismutase, and catalase as well as levels of lipid peroxidase that are related to the health and immunity of *Betta* sp.

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