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Terminalia catappa L. extract improves survival, hematological profile and resistance to *Aeromonas hydrophila* in *Betta* sp.

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Abstract. To determine the effects of *Terminalia catappa* extract (TCE) immersion on the survival and hematological profile of *Betta* sp, a group of fish was immersed in 500 ppm TCE and compared with the control group. After three days of immersion, survival, leukocyte (WBC), erythrocyte (RBC), hemoglobin (Hb), hematocrit (Hct), platelet (PLT), and differential leukocyte counts were compared between the two groups. Surviving fish from each group were then divided into three subgroups: subgroup without injection; subgroup injected with normal saline; subgroup injected with *Aeromonas hydrophila*. Survival, WBC, RBC, Hb, Hct, PLT, the percentage and number of lymphocyte, monocyte, and granulocytes post injection were evaluated for 48 h. The results showed that the survival of immersed fish was significantly higher than that in the control. No significant differences in the hematological profile were noted between the control and the immersed fish. The WBC of control subgroup (*A. hydrophila* injection) was significantly increased after 24 h. The fish immersed and injected with *A. hydrophila* had the highest PLT. The number of lymphocytes of all subgroups of fish was stable while the percentage of monocytes and granulocytes of the subgroups of immersed fish were increased. This finding suggested that 500 ppm of TCE is beneficial for improving survival, blood profile, and resistance to *A. hydrophila*.

Keywords: *Terminalia catappa*, survival, blood profiles, *Betta* sp., *Aeromonas hydrophilla*

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Introduction

Aeromonas species have been identified as causative agents and serious pathogens to various aquatic animals (Carriero et al. 2016, Maliwat et al. 2016, Ni et al. 2016, Yang et al. 2016) resulting in mass mortalities to many cultured ornamental fish (Majtán et al. 2012, Abdi et al. 2014, Harikrishnan et al. 2015). Among various *Aeromonas* species, *Aeromonas hydrophila* is known to cause disease outbreaks in Labeo rohita (Hamilton) (Das et al. 2015), goldfish, *Carassius auratus* (L.) (Anusha et al. 2014, Sahoo et al. 2016), and red hybrid tilapia (*Oreochromis* spp.) (Kirubakaran et al. 2016, Lee et al. 2016, Musthafa et al. 2016). Intramuscular injection of *A. hydrophila* can produce a virulent reaction and reduce immune responses in *Pangasianodon hypophthalmus* (Sauvage) (Tamamdusturi and Yuhana 2016). To overcome high mortalities of cultured animals, aquaculturists and researchers use antibiotics to prevent virulent reactions to *A. hydrophila* infection (Guz and Kozinska 2004, Sinclair et al. 2016).

However, increasing global demand for the preservation of eco-friendly environments, the application of antibiotics, which are notorious for increasing antibiotic-resistant pathogens and inducing environmental deterioration, is being questioned (Samanidou and Evaggelopoulou 2007, He et al. 2016, Uchida et al. 2016). Alternatively, various

plant extracts, such as oat (*Avena sativa*), oyster mushroom (*Pleurotus ostreatus*), nettle (*Urtica dioica*), sea grass (*Cymodocea serrulata*), turmeric (*Curcuma longa*), spirulina (*Spirulina platensis*), and beet root (*Beta vulgaris*) have been tested and used as alternatives to antibiotics (Baba et al. 2016, Bilen et al. 2016, Devi et al. 2016). There has been a surge in the use of plant extracts to enhance the survival and immune competence of cultured aquatic animals (Reverter et al. 2014, Adel et al. 2016, Kakaei and Shahbazi 2016).

One potential plant that can be used as an antimicrobial to enhance survival and immunocompetence is the Indian almond tree (*T. catappa*). Terminalia species are widely distributed in both tropical and sub-tropical regions, including in Asian (Hyttel et al. 2010). In Indonesia, *T. catappa* L. is an attractive, long-lived tree well suited to ornamental and amenity plantings. The water extract of *T. catappa* leaf is well known as a folk medicine for antipyretic, hemostatic, hepatitis, and liver-related diseases in the Philippines, Malaysia, and Indonesia (Meena and Raja 2006, Vučurović and Razmovski 2012).

According to Rakholiya and Chanda (2012), *T. catappa* extract (TCE) shows the highest and strongest synergistic effect against the bacterial strains tested. Meanwhile, a study in fish revealed that TCE can be used as an alternative antibacterial remedy against tilapia, *Oreochromis niloticus* (L.) parasites and bacterial pathogens (Öztop et al. 2002, Goh et al. 2010). According to Pandey (2013), TCE is also a potential plant biomedicine for improving the non-specific defense mechanism in fish and elevating the specific immune response. The use of TCE in *O. niloticus* (Chitmanat et al., 2003) and post-larval black tiger shrimp, *Penaeus monodon*, (Ikhwanuddin et al. 2014) also improved their survival and immunity. Further, TCE also increased the hematological profiles of African catfish, *Clarias gariepinus* (Burchell) (Julius et al. 2015). Additionally, TCE with phytochemical active ingredients such as flavonoid is useful for boosting immune function and an antioxidant enzyme that is responsible for preventing cellular damage and improving immune competence (Middleton and Kandaswami 1992, Middleton 1998, Saroja et al. 2012).

The immune status of fish is related to their hematological parameters, which are important in fish culture because of their value in monitoring the health status of fish (Chandel et al. 2009). Hematological parameters such as red blood cell (RBC), hemoglobin count (Hb), hematocrit (Hct), platelet (PLT), and total and differential leukocyte counts (TLC and DLC) in fish are effective tools that can be used to evaluate physiological and pathological changes in fish (Inama et al. 1993). Leukocyte counts are used regularly as indicators of fish health status because leukocytes are key components of innate immune defense and are involved in the regulation of immunological function in fish (Zhou et al. 2010, Ekman et al. 2013). Moreover, immunity related physiological responses measured by alterations in TLC and DLC can be used as indicators of immune competence and the health status of several fish species (Alvarez et al. 1988, Modrá et al. 1998, Do Huu et al. 2016).

The ornamental fish *Betta* sp. is a member of the Labyrinth fish family (Belonitiidae) and is known as a colorful species, especially in males (Ekman et al. 2013). As an important cultured ornamental species in Indonesia, *Betta* sp. is recognized as a substantial ornamental fish commodity (Alfian 2010). Increasing market demand for this fish has led to a significant boost in research to improve the survival and health performance of it.

However, information regarding the effects of TCE immersion on the survival and immune competence of ornamental fish when challenged with *A. hydrophila* is unknown. Thus, the current study was designed to evaluate the effects of TCE immersion on survival, RBC, Hb, Hct, PLT, TLC, and DLC, including the number and percentage of lymphocytes, monocytes, and granulocytes in *Betta* sp. when challenged with *A. hydrophila*.

Materials and methods

Plant material

Dried, cut brown *T. catappa* leaves were collected on the campus of Mulawarman University, Barong Tongkok, East Kalimantan, Indonesia. To eliminate extraneous matter, the collected *T. catappa* leaves were washed with deionized water and immediately dried in an oven at 40°C for 12 h. *T. catappa* powder was obtained using a mill. The powder was extracted with ethanol 95% for 3 days (100 g per 100 mL). After filtration, the extract was evaporated using a rotary evaporator and stored at 4°C until it was used as a crude extract.

To detect the presence of possible phytochemicals in the crude extract of *T. catappa* leaves, some preliminary phytochemical tests for alkaloid, saponin, steroid, triterpenoid, quinon, phenolic, tannin, and flavonoid were performed as follows: (1) Alkaloid-Dragendorff Test – 2 mL of the filtrate was added to 1 mL of Dragendorff reagent along the side of the test tube, an orange or orange-reddish-brown precipitate indicated a positive test, (2) test for saponin – 1 mL of extract + 5 mL distilled water was shaken vigorously, the appearance of stable froth for 15 minutes indicated the presence of saponin, (3) test for steroids and (4) triterpenoid (Liebermann-Burchard): – 2 mL extract + 1 mL chloroform + a few drops of acetic anhydride + concentrated sulphuric acid added along the side of the test tube, the appearance of a blue or green color indicated the presence of steroids, while the appearance of a red or brown color indicated the presence of triterpenoid, (5) test for Quinon – 1 mL of extract + 1 mL of concentrated sulfuric acid, a red color indicated the presence of quinon, (6) test for phenols and tannins – crude extract was mixed with 2 mL of 2% solution of $FeCl_3$, a blue-green or black color indicated the presence of phenols and tannins, (7) test for flavonoids – 2 mL extract + concentrated hydrochloric acid + magnesium ribbon, the appearance of pink-red color indicated the presence of flavonoids.

Animals and experimental setup

Three hundred and sixty two-month old fish (average initial weight 0.62 g) were purchased from a local breeding farm in Samarinda, East Kalimantan (Indonesia) and acclimated at the Animal Physiology, Development, and Molecular Laboratory, Mulawarman University, East Kalimantan for three days. The fish were then randomly distributed into two groups (C and P) with triplicate groups of twenty fish per replicate group. Each fish was then placed in an individual glass tank (0.5 L capacity, 0.4 L of freshwater in each tank). For three days, 500 ppm TCE was added to the fish in group P (Immersion), while there was no immersion in group C (Control). Temperature, pH, and dissolved oxygen (DO) were measured daily with a thermometer and a HM-7 pH meter (TOA-DKK Corporation, Japan). Nitrate, nitrite, and ammonium were measured and recorded weekly using chemical test kits (Aquarium Pharmaceuticals™, Inc., USA). The fish in each glass tank was fed with frozen blood worm at a ratio of 1% of body weight every day. The optimum immersion (500 ppm) and feeding rations were determined during previous experiments (Nugroho et al. 2016). Uneaten food and feces were siphoned out before renewing the water. The water volume was renewed every day at a concentration of 500 ppm (only for group P) and maintained at 0.4 L of water in every glass tank.

Challenge test

At the end of the trial, both groups of fish (C and P) were further divided into three triplicate subgroups each (20 fish per replicate). Two subgroups, one from each group, were injected with 20 µL of *A. hydrophila* stock suspension (10^6 CFU mL^{-1}) that was obtained from the Department of Biology, Faculty of Mathematics and Natural Sciences, Mulawarman University, Indonesia; two subgroups from each group were injected with 20 µL normal saline solution and; the third and final two subgroups from each group were not injected (control subgroup). All injections were performed

intraperitoneally. All fish were then monitored for survival, RBC, Hb, Hct, PLT, TLC, and DLC, including the number and percentage of lymphocytes, monocytes, and granulocytes at 0, 24, and 48 h post-injection time.

Sampling and analytical procedure

The survival rate of the fish in each group was recorded daily during the pre-challenge test and every 24 h post injection for 48 hours. At day three after immersion and every 24 h interval during the challenge test, blood samples were taken by caudal venipuncture after anesthetizing 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. Hemoglobin (Hb) was determined according to the cyanmethemoglobin procedure (Blaxhall and Rao 1973) and expressed in g dL^{-1}. The number and percentage of lymphocytes, monocytes, granulocytes, Htc, and PLT were determined using an Mindray BC2800 Auto Hematology Analyser (Mindray® Shenzhen, China).

Statistical analysis

Results are expressed as means ± standard error (SE) and the data were analyzed using SPSS version 21 (SPSS, Inc., USA). The data on survival, the percentage of lymphocytes, monocytes, and granulocytes were transformed to arcsine. The WBC, RBC, Hb, the number of lymphocytes, monocytes, and granulocytes on day three after immersion were subjected to the T-test to evaluate the significance of differences among groups. Meanwhile, survival, WBC, RBC, Hb, Htc, PLT, the percentage and number of lymphocytes, monocytes, and granulocytes during the challenge test were subjected to two way ANOVA, followed by Tukey's post hoc test to evaluate significant differences among the groups of treatments. All tests were significant at $P < 0.05$.

Results

The screening test for bioactive compounds in TCE must be done to determine the benefit of phytochemical bioactive agents in fish. The current study showed the presence of saponin, triterpenoid, quinon, phenolic, tannins, and flavonoid in the TCE (Table 1).

Table 1
Phytochemical screening test of *T. catappa* leaf extract. (+) Present; (-) Absent

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

During the three days of the immersion treatment, the temperature, pH, DO, nitrate, nitrite, and ammonia levels were recorded as follow: temperature – 27.9°C ± 0.3, pH – 7.37±0.2, and dissolved oxygen level – 8.2 ± 0.3 mg L^{-1}. The nitrate, nitrite, and ammonia levels in the water of the control and 500 ppm TCE treatment groups were below the limits of detection.

The survival and hematological profiles of the control group and the group of fish immersed in 500 ppm TCE are presented in Table 2. After three days of immersion, the survival of fish in the TCE immersion was significantly higher (T-test, $P < 0.05$) than in the control group, whereas blood parameters such as WBC, RBC, Hb, Htc, PLT, and the number of lymphocytes, monocytes, and granulocytes were not affected by 500 ppm TCE immersion.

After being challenged with *A. hydrophila*, the survival rates of fish without immersion were significantly lower ($P < 0.05$) than those of the other fish

Table 2
Mean ± SE (standard error) survival and blood profile of *Betta* sp. after three-day immersion in 500 ppm *T. catappa* leaf extract

	Groups	
Parameters	Control	500 ppm
Survival (%)	$88.88^{a} \pm 0.5$	$98.88^{b} \pm 2.42$
WBC (10^3 μL^{-1})	8.32 ± 0.21	8.38 ± 0.12
RBC (10^6 μL^{-1})	1.64 ± 0.14	1.72 ± 0.04
Hb (g dL^{-1})	6.22 ± 0.46	6.11 ± 0.38
Htc (%)	18.66 ± 1.40	16.66 ± 0.47
Lymphocytes (10^3 μL^{-1})	7.00 ± 1.01	5.00 ± 0.23
Monocytes (10^3 μL^{-1})	1.22 ± 0.22	1.66 ± 0.23
Granulocytes (10^3 μL^{-1})	1.77 ± 0.22	1.88 ± 0.20
PLT (10^3 μL^{-1})	34.88 ± 1.04	35.77 ± 1.81

WBC – White blood cell; RBC – Red blood cell; Hb – Hemoglobin; Htc – Hematocrit; PLT – Platelet. Different letter indexes (a, b) indicate significantly different means for different treatments at $P < 0.05$.

(Fig. 1). The WBC counts of the control fish increased significantly after the challenge with *A. hydrophila*. The highest WBC count ($12.36 \pm 0.19 \times 10^3$ μL^{-1}) was noted in the control fish subgroup at 48 h post challenge (Table 3). Meanwhile, the PLT of TCE-immersed fish that were injected with the *A. hydrophila* pathogen was significantly the highest ($P < 0.05$) at 24 h post challenge, followed by a decline in the number of PLT at 48 h post challenge. The RBC, Hb, and Hct of all subgroups of fish were not significantly different during the challenge test.

During the challenge test, the 500 ppm TCE-immersed fish had the lowest percentage of lymphocytes. However, the highest percentage of monocytes and granulocytes were found in the subgroups of infected fish that had been immersed in

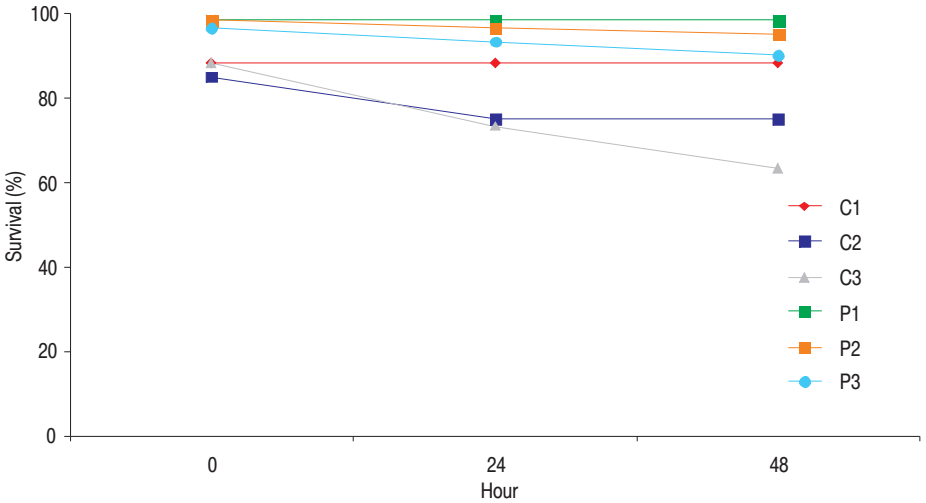

Figure 1. Survival of *Betta* sp. challenged with *A. hydrophila*. Note: C1 – control with no injection; C2 – control with 20 µL normal saline injection; C3 – control with 20 µL *A. hydrophila* injection; P1 – 500 ppm *T. catappa* leaf extract (TCE) immersion with no challenge; P2 – 500 ppm immersion with 20 µL normal saline injection; P3 – 500 ppm immersion with 20 µL *A. hydrophila* injection. *Significant difference at $P < 0.05$.

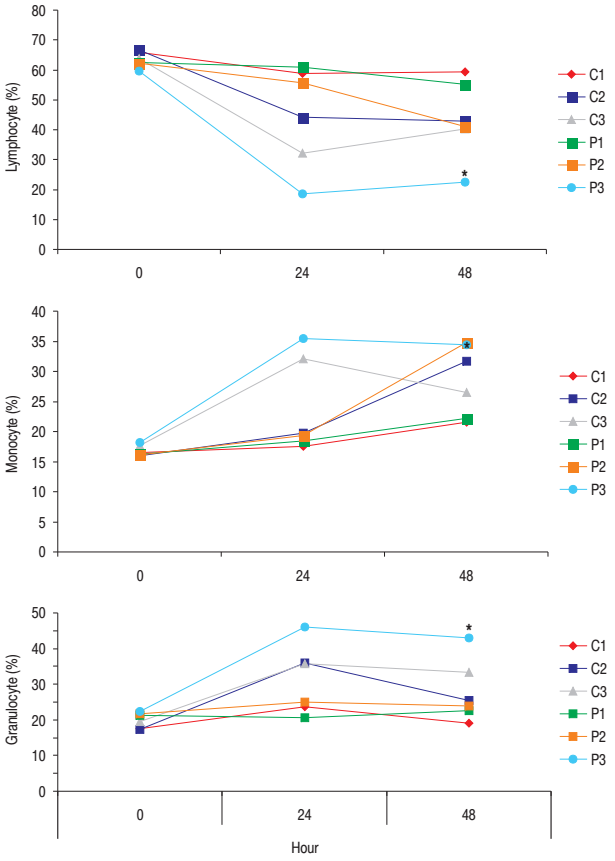

Figure 2. Lymphocyte, monocyte, and granulocyte count percentages in the blood of *Betta* sp. Note: C1 – control with no injection; C2 – control with 20 µL normal saline injection; C3 – control with 20 µL *A. hydrophila* injection; P1 – 500 ppm *T. catappa* leaf extract (TCE) immersion with no challenge; P2 – 500 ppm immersion with 20 µL normal saline injection; P3 – 500 ppm immersion with 20 µL *A. hydrophila* injections. *Significant difference at $P < 0.05$.

500 ppm TCE (Fig. 2). The number of lymphocytes of all subgroups of fish was stable during the challenge test, whereas the number of monocytes and granulocytes of all the subgroups of fish were significantly increased 24 h post challenge. The highest number of monocytes and granulocytes were noted in the TCE-immersed fish challenged with *A. hydrophila* 48 h post injection (Fig. 3).

Discussion

Over the past few years, infectious diseases caused by *A. hydrophila* have become a major problem in fish culture causing heavy economic losses because of high mortalities. Plant-based extracts have been proven to enhancesurvival and immunocompetence in cultured fish. A number of plant extracts that have active ingredients and various biological activities have been reported as suitable for use as supplements in aquaculture (Citarasu 2010, Madhuri et al. 2013, Chakraborty et al. 2014, Sivasankar et al. 2015, Syahidah et al. 2015). The current study indicates that the application of 500 ppm TCE, which contains active phytochemical compounds such as saponin, triterpenoid, quinon, phenolic, tannins, and flavonoid, can significantly improve the survival of *Betta* sp. even when they are challenged with *A. hydrophila*. Similarly, improved survival of *A. hydrophila*-infected goldfish (*C. auratus*) fed herbal plant extract (*Ixora coccinea*) has also been reported (Anusha et al. 2014).

Plant-based bioactive compounds are mostly biodegradable in nature, ecofriendly, safe for humans, biocompatible, and usually less expensive than synthetic compounds (Immanuel et al. 2009). Several phytochemical compounds such as saponin, triterpenoid, quinon, phenolic, tannins, and flavonoid are found in various parts of some plants, including the leaves of *T. catappa* (Citarasu 2010, Chakraborty et al. 2014). The phytochemicals which are contained in the plant may boost the innate immune system and could be used widely in fish culture (Chakraborty and Hancz 2011). Some previous studies have been conducted to determine the use of phytochemicals from various parts of plant extracts to increase survival rates of marine ornamental fish (Balachandran and Tissera 2013), Nile tilapia (*O. niloticus*) (Abdel-Tawwab et al. 2010, Akinwande et al. 2011) and common carp, *Cyprinus carpio* L. (Soltanian and Fereidouni 2016) against *A. hydrophila*.

Survival

The current study found that immersing fish for three days in TCE and the subgroups of TCE-immersed fish with or without injections exhibited significantly higher ($P < 0.05$) survival rates than did the control

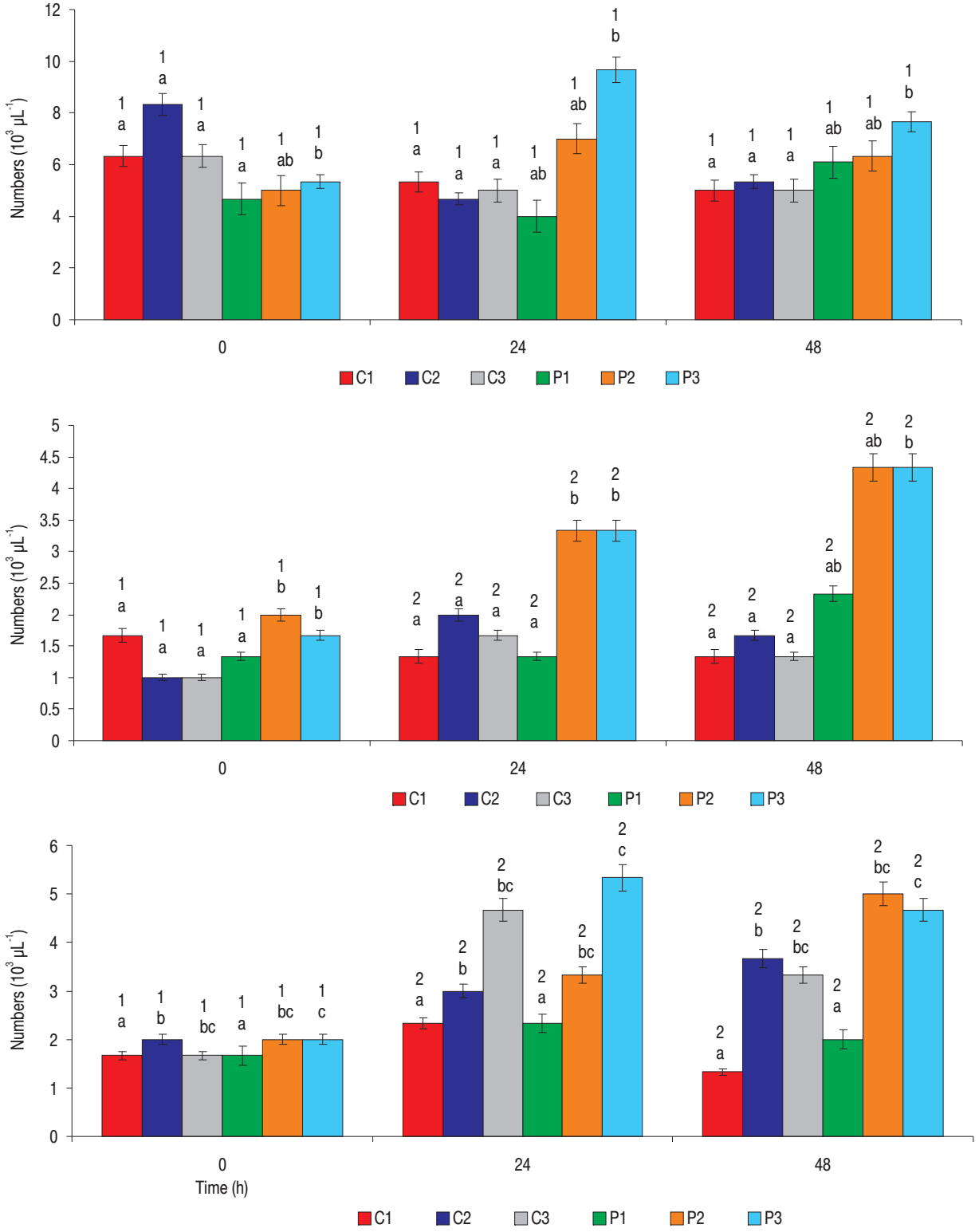

Figure 3. Lymphocyte, monocyte, and granulocyte count numbers in the blood of *Betta* sp. challenged with *A. hydrophila*. Different letter indexes (a, b, c) over bars indicate significantly different means for different treatments at $P < 0.05$. Different numerical indexes (1, 2, 3) over bars indicate significantly different means at different times at $P < 0.05$. Note: C1 – control with no injection; C2 – control with 20 µL normal saline injection; C3 – control with 20 µL *A. hydrophila* injection; P1 – 500 ppm *T. catappa* leaf extract (TCE) immersion with no challenge; P2 – 500 ppm immersion with 20 µL normal saline injection; P3 – 500 ppm immersion with 20 µL *A. hydrophila* injections.

Table 3
Mean ± SE blood profile of *Betta* sp. challenged with *A. hydrophila*

		Groups					
		Control			500 ppm		
Parameters	Hour	C1	C2	C3	P1	P2	P3
WBC (10^3 μL^{-1})	0	8.00 ± 0.50^{a1}	8.43 ± 0.31^{b1}	8.53 ± 0.43^{c1}	8.13 ± 0.32^{a1}	8.13 ± 0.27^{a1}	8.03 ± 0.34^{a1}
	24	8.50 ± 0.18^{a2}	10.43 ± 0.42^{b2}	11.70 ± 0.41^{c2}	8.43 ± 0.22^{a2}	8.56 ± 0.22^{a2}	8.36 ± 0.22^{a2}
	48	8.33 ± 0.19^{a2}	9.13 ± 0.44^{b2}	12.36 ± 0.19^{c2}	8.43 ± 0.13^{a2}	8.90 ± 0.25^{a2}	8.86 ± 0.22^{a2}
RBC (10^6 μL^{-1})	0	1.43 ± 0.13^{a1}	1.83 ± 0.50^{a}	1.66 ± 0.27^{a}	1.70 ± 0.18^{a}	1.76 ± 0.22^{a}	1.70 ± 0.25^{a}
	24	1.76 ± 0.26^{a}	1.80 ± 0.18^{a}	1.66 ± 0.19^{a}	1.76 ± 0.22^{a}	1.70 ± 0.18^{a}	1.73 ± 0.13^{a}
	48	1.66 ± 0.13^{a}	1.73 ± 0.13^{a}	1.73 ± 0.22^{a}	1.80 ± 0.18^{a}	1.63 ± 0.22^{a}	1.66 ± 0.13^{a}
Hb (g dL^{-1})	0	6.00±1.41a	6.66±1.49a	6.00 ± 1.41^{a}	6.33 ± 1.45^{a}	5.33 ± 1.33^{a}	6.66 ± 1.49^{a}
	24	6.33 ± 1.45^{a}	5.33 ± 1.33^{a}	6.33 ± 1.45^{a}	6.66 ± 1.49^{a}	5.66 ± 1.37^{a}	6.33 ± 1.45^{a}
	48	6.66 ± 1.49^{a}	6.66 ± 1.49^{a}	6.00 ± 1.41^{a}	7.00 ± 1.52^{a}	5.66 ± 1.37^{a}	6.33 ± 1.45^{a}
Hct (%)	0	16.33 ± 0.91^{a}	20.66 ± 1.53^{a}	19.00 ± 1.57^{a}	15.66 ± 0.43^{a}	17.66 ± 0.71^{a}	16.66 ± 0.71^{a}
	24	18.33 ± 0.83^{a}	17.00 ± 0.57^{a}	17.00 ± 0.75^{a}	18.33 ± 0.43^{a}	17.00 ± 0.57^{a}	17.33 ± 0.71^{a}
	48	16.66 ± 0.62^{a}	18.00 ± 0.57^{a}	17.33 ± 0.71^{a}	16.66 ± 0.43^{a}	18.33 ± 0.43^{a}	16.66 ± 0.43^{a}
PLT (10^3 μL^{-1})	0	36.66 ± 1.20^{a1}	34.00 ± 0.93^{a1}	34.66 ± 1.03^{a1}	33.00 ± 0.57^{a1}	40.00 ± 1.20^{b1}	34.33 ± 1.58^{b1}
	24	41.66 ± 1.78^{a2}	32.00 ± 1.47^{a2}	40.00 ± 1.07^{a2}	33.33 ± 0.43^{a2}	46.00 ± 1.81^{b2}	49.33 ± 1.28^{b2}
	48	31.66 ± 0.71^{a1}	32.00 ± 0.75^{a1}	33.33 ± 0.71^{a2}	31.66 ± 0.98^{a1}	33.00 ± 0.57^{b1}	34.00 ± 0.57^{b1}

Different letter indexes (a, b, c) indicate significantly different means for different treatments at $P < 0.05$. Different numerical indexes (1, 2) indicate significantly different means at different times at $P < 0.05$. Note: WBC – White blood cell; RBC – Red blood cell; Hb – Hemoglobin; Htc – Hematocrit; PLT – Platelet. C1 – control with no injection; C2 – control with 20 μL normal saline injection; C3 – control with 20 μL *A. hydrophila* injection; P1 – 500 ppm *T. catappa* leaf extract (TCE) immersion with no challenge; P2 – 500 ppm TCE immersion with 20 μL normal saline injection; P3 – 500 ppm TCE immersion with 20 μL *A. hydrophila* injection.

group. Similar results reported on previous work indicate that Nile tilapia immersed in extracts of *Asparagus racemosus* (Mukherjee et al. 2015) and *Neobenedenia* sp. with *Allium sativum* (Militz et al. 2014) had significantly better survival than did controls without immersion. The high survival percentage of fish in different treatments could indicate that the phytochemical content in the plant extracts contributed to improved fish health.

Phytochemical extracts from various parts of plants were shown to enhance survival rates in common carp (*C. carpio*), marine ornamental fish (Balachandran and Tissera 2013), and tilapia (*O. niloticus*) (Akinwande et al. 2011). Plants, especially *T. catappa*, are rich in a wide variety of secondary metabolite phytochemicals such as tannins and saponin (Citarasu 2010, Nugroho et al. 2016). Cultured post-larval black tiger shrimp (*P. monodon*) treated with the tannins in TCE had significantly higher survival rates (Ikhwanuddin et al. 2014). According to Van-Sumere et al. (1975) and Ashraf and Bengston (2007), tannin can absorb harmful chemicals and provide a soothing, suitable environment that is fairly benign for fish. Besides tannin, the

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

Blood Profile

Determining how phytochemicals are related to hematological indices is common in the culture of aquatic animals, including fish. Hematological parameters are used as indicators of the response of fish health status, especially in the presence of disease and under stressful conditions (Osman et al. 2010, Karimi et al. 2013, Suely et al. 2016). Hematological indices such as WBC, RBC, Hb, the number and percentage of lymphocytes, monocytes, and granulocytes play important roles in assessing the physiological condition of fish.

Based on the current results, it was found that the WBC count of control subgroups of fish challenged with *A. hydrophila* had significantly increased 24 h post injection, whereas the number of WBC of all the subgroups of immersed-fish were not significantly different. Meanwhile, the RBC, Hb, and Hct of all the subgroups of fish during the challenge test were not affected. These results are in agreement with previous findings that indicate there was an increasing number of WBC in Nile tilapia after infection (Martins et al. 2009). Pourmoghim et al. (2015) also found similar results in that dietary *Origanum vulgare* extract incorporated into test diets had no significant effect on RBC, WBC, Hct, hemoglobin, or Hb.

The underlying mechanism(s) whereby the WBC of TCE-immersed fish did was not altered is not properly understood. However, Rattanachaikunsopon and Phumkhachorn (2010) and Reverter et al. (2014) reported that flavonoids from plant metabolites may contribute to antibacterial activity. Flavonoids contained in TCE were able to inhibit bacterial growth. Thus, it is clear that the WBC counts of the subgroup of fish immersed in TCE did not change. Additionally, flavonoid has a positive effect in reducing RBC hemolysis which can be caused by bacterial infection, by protecting the biological membranes of the RBC (Kitagawa et al. 1992, Asgary et al. 2005). Some investigators also report that the antioxidants present in plant extracts might trigger erythropoiesis. This seems to be in agreement with the present study as TCE has flavonoid that, as an antioxidant, might maintain the heme iron in its ferrous state (Akah et al. 2007, Uboh et al. 2010, Shatoor 2011).

Besides total WBC, differential WBC such as lymphocytes, monocytes, and granulocytes produce antibodies to acknowledge and respond to the antigen and to act as mediators of cellular and humoral immune responses (Abbas et al. 2010, Soltanian and Fereidouni 2016). The current results found that fish immersed in 500 ppm TCE had the lowest percentage of lymphocytes but the highest percentage of monocytes and granulocytes. The number of lymphocytes of all the subgroups of fish was stable during the challenge test, whereas the number of monocytes and granulocytes of all the subgroups of fish were significantly increased 24 h post challenge. The highest number of monocytes and granulocytes were noted in the TCE-immersed fish challenged with *A. hydrophila* 48 h post injection. The significant increase in monocytes and granulocytes in this study is in agreement with Gabriel et al. (2015) who reports that the percentage of monocytes and granulocytes in tilapia (GIFT) infected with Streptococcus iniae increased after the challenge test. Additionally, Aly et al. (2015) report that Nile tilapia vaccinated with *A. hydrophila* had no significant different lymphocyte values compared to unvaccinated Nile tilapia.

The increase in monocyte and granulocytes in TCE-immersed fish can be attributed to the improvement of non-specific immune responses. However, the number of lymphocytes that was stable during

the challenge test may indicate that there was no enhancement in specific immune induction. Monocytes play a pivotal role in the immune defense system of fish. They can transform into macrophages and exhibit phagocytosis activity against pathogens at first recognition and subsequent infections. Granulocytes, such as neutrophils, are the primary cells responsible for first infections and the phagocytosis of bacterial pathogens during challenge tests (Sivagurunathan et al. 2011).

Conclusions

In summary, this study indicates that 500 ppm TCE immersion can potentially be used to combat *A. hydrophila* as it increases the survival and hematological profile, such as the number and percentage of monocytes and granulocytes, of *Betta* sp. The study has also shown that the application of TCE might also be of practical use in disease management strategies in fish, especially the ornamental fish *Betta* sp. Further research needs to be conducted to examine the lysozyme activity, respiratory burst, and the antioxidant profiles of fish immersed in 500 ppm TCE.

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