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Efficacy of mangosteen (*Garcinia mangostana*) peel hot water extract against *Aeromonas hydrophila* infection of seabass fingerling (*Lates calcarifer*)

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Abstract The mangosteen (*Garcinia mangostana* Linn.) peel hot-water extract (MPE) is proved to be antibacterial potential of seabass fingerling (*Lates calcarifer*) rearing in freshwater which infected by *Aeromonas hydrophila*. *In vitro* study, Minimal Inhibitory Concentration (MIC) of MPE was 25 ppm and the Minimal Bactericidal Concentration (MBC) was 25 ppm. For *In vivo*, fingerlings were immersed in different concentrations of MPE by 0 ppm (Control), 20 ppm, 40 ppm, and 60 ppm respectively for 7 days with *A. hydrophila* concentration of 10^8 CFU mL^{-1}. The results showed that the group which received MPE were higher survival rate compare with control group. Hematological parameters revealed that the group that received MPE had significantly increased levels of red blood cells (RBC), white blood cells (WBC), and hemoglobin concentration (Hb) than control group. Moreover, the water quality parameters were not significantly different except ammonia concentration, at 60 ppm MPE concentration of ammonia was the lowest. All results can imply that the MPE is able to improve the antibacterial potential and culture potential for seabass fingerlings.

Keywords: Mangosteen peel extract, *Aeromonas hydrophila*, Antimicrobial, Seabass

Introduction

The most popular fish for aquaculture in Thailand is seabass (*Lates calcarifer*), which cultured in both freshwater and brackishwater. According to Department of Fisheries Thailand, the statistics of mariculture farm in 2022, seabass was 95.97 % culture areas of marine fish farms (DOF, 2022). Since seabass is an euryhaline fish which capable of adapting to a wide range of salinities, it is an effective option for farms that are distant from the sea or

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terrestrial farms where it is difficult to find seawater. The cultivation seabass in freshwater seems to be effective and simple to manage (Khang *et al*., 2018). Due to the high output of fry and the high rate of customer demand, seabass cultured in freshwater appeared to have more average than in the past. However, the seabass farming industry continues to struggle with a pandemic of disease and stress that might lead to aquatic animal sickness. The most concern factor for farmer is pathogenic. One of that is a bacteria infection, which has the power for severe damage to the culture system (Toranzo *et al*., 2005). The bacteria that able to occur in seabass cultured is *Aeromonas hydrophila*, which regularly causes harm to commercial freshwater species like catfish and tilapia, this bacteria lives in fish from fry to adult and spread through contaminated waterways (Austin and Austin, 1999) *A. hydrophila* is a gram-negative bacterium which is facultative anaerobic, rod-shaped and belongs to the *Vibrionaceae* family (Inglis *et al*., 1993). When infected by *A. hydrophila*, the external symptoms include hemorrhagic septicemia, infectious abnormal dropsy, exopthalmia, fin, and tail rot. Internal symptoms include anemia and organ damage particularly in the kidney and liver (Austin and Austin, 1999). hen fish invaded by these bacteria, they become weak and difficult to cure. They have several methods for solving the bacteria infection problem by improving farming quality, nutritional, water quality, and reducing fish density. Use of antibiotics to cure the sickness or immerse the fish in chemicals are an effective method (Alderman and Smith, 2001). However, antibiotics and chemicals have disadvantages include drug resistance, environmental accumulation, and contamination. According to the above, we have to find the new method for disease prevention that is environmentally friendly, effective, and practical. Nowadays, adding value to agricultural bio-waste is a popular technique to reduce waste (Verissimo *et al*., 2021).

This was an interesting, value addition to fruit by-product. Mangosteen (*Garcinia mangostana* Linn.) is a tropical fruit of the *Guttiferae* family. Mangosteen is a well-known fruit by their color, look, and the most importantly, delicious flavor. Mangosteen might be considered as the "Queen of Fruit". Southeast Asian countries include India, Myanmar, Philippines, Sri Lanka, and Thailand cultivate it (Pedraza-Chaverri *et al*., 2008). Mangosteen peel is always use as traditional medicine to treat diarrhea, inflammation, and chronic sores (Suksamrarn *et al*., 2006). Some people claimed that mangosteen contains xanthone, terpenoids, and sugar from various parts of the plant (peel, fruit, bark, and leaf). Mangosteen also has an anticancer, antibacterial, antifungal, anti-inflammation, and antioxidant properties (Soosean *et al*., 2010). The mangostin, xanthone, tannin, garcinone, and tovophyllin are some of the biochemical compounds included in mangosteen peel (Husen *et al*., 2017). In aquaculture, it

was found that when catfish received mangosteen shoot extract for 35 days, the extract had no effect on growth performance but able to increased hematological parameters (Suksamrarn *et al.,* 2006). When application mangosteen peel extract to against *Vibrio harveyii* in grouper found that the mangosteen peel extract was effectiveness (Akmal *et al*., 2021). Moreover, injected or used the mangosteen husk hot-water extract as diet supplement to *Macrobranchium rosenbergii*. The prawns which received mangosteen husk hot-water extract have an increased in immune parameters, stress reduction, and enhancing resistance to *Lactococcus garvieae* (Kitikiew *et al*., 2023; Kuo *et al*., 2023).

The objective was to use hot-water extract from the peel of mangosteen (*G. mangostana* L.) against *A. hydrophila* in seabass (*L. calcarifer*), to determine antibacterial efficacy by using Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC), hematological parameters as a diagnostic tool for monitoring health status of fish and infected status (Fazio, 2019) following by histopathological analysis to observe the tissues.

Materials and methods

Experimental design

The seabass (body weighs are 5.88 ± 0.21 g and body length are 9.17 ± 0.21 cm) obtained from Chachengsao province, Thailand. The fish were acclimation for 2 weeks at salinity 0 ppt, 25-28 ℃. Feeding the fish by commercial feed which content 42% protein, 2 times a day until satiation. After 2 weeks, the 20 fish were randomly put in each 8 tanks with 30 L of freshwater and aeration system at 28 ℃. *A. hydrophila* suspension concentration of 10^8 CFU mL^{-1} was challenged to fish by bath exposure (Bromage and Owens, 2002) for 1 h. MPE was given after the bacteria challenged which different concentration: 0 ppm (Control group: C), 20 ppm (G20), 40 ppm (G40), and 60 ppm (G60). Used Completely randomized design with 4 treatments. The fish were reared for 7 days with feeding until sanitation and without water changed.

Preparation of mangosteen peel hot-water extract (MPE)

Mangosteen peels were obtained from Chanthaburi province, Thailand. The raw peel was cleaned by sterile water and dried the peel at 50 ℃ for 3 days. After that, ground into powder and sieving by No. 80 mesh sieve. Mangosteen peel powder 100 g was added by 1 L of sterile water, heated on hot plate at 95 ℃ for 10 minutes and let it cool at room temperature. Centrifuged the extract at

6000 rpm for 20 minutes. Extract kept at -60 ℃ for 1 day, use freeze-dried for 72 h. The MPE was obtained.

Preparation of Aeromonas hydrophila

A. hydrophila obtained from laboratory of Fisheries Science Department, King Mongkut's Institute of Technology Ladkrabang (KMITL). Bacteria cultured on nutrient agar (NA) for 24 h at 30 ℃ before transferred to nutrient broth (NB) and cultured for 16 h at 30 ℃. The medium was centrifuged at 1200 rpm at 4 ℃ for 15 minutes the NB was eliminated, and bacterial pellet was re-suspended at 10^8 CFU mL^{-1} for challenging test.

Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Minimal Inhibition Concentration (MIC) of MPE was performed by culture *A. hydrophila* (10^8 CFU mL^{-1}) in NB 1 mL with different MPE concentration (50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 ppm) by serial dilution. After inoculated, NB with bacteria was incubated at 30℃ for 24 h. The 10 µL of NB without turbidity transfered to the NA and incubated at 30℃ for 24 h for Minimal Bactericidal Concentration (MBC) test (Wiegand *et al*., 2008).

Survival rate and clinical symptoms observation

After challenged the mortality was observed every day. The died fish represent clinical symptoms to verified the cause of dead. Survival rate (SR) calculated by formula below (Ali *et al*., 2017).

Survival rate (%) = (Final number of fish / Initial number of fish) x 100

Hematological parameters collection

At 0, 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th}, and 7^{th} day after challenged, the blood collected from 3 fish in each group by needle and syringe coated by 3% EDTA solution as an anticoagulant to collect the blood from the caudal vein. The blood was separate into 2 parts. The first part, 5 µL of blood was transferred into 995 µL Natt-Herricks stain solution (1:200 diluted) for red blood cell counting (RBC) and white blood cell counting (WBC). And another part, 5 µL of blood transferred into 1.23 mL Drabkin's solution for hemoglobin concentration (Hb) determination. RBC and WBC performed by directed count under microscope (Nikon ECLIPSE E200) by hemocytometer and give 10 µL of stained blood into

slide gently. Hb were determinated by cyanomethemogoblin method. (Blaxhall and Daisley, 1973).

Histopathology

The liver and kidney samples of fish were collected from unchallenged group and challenged group: 0 ppm (Control group: C), 20 ppm (G20), 40ppm (G40), and 60ppm (G60). The tissues were fixed in Davidson's fixative (Fournie *et al*., 2000). The tissues were dehydrated by different concentration series of alcohol in tissue processor (Leica TP 1020), after dehydration the tissues were embedding in paraffin on the cassette box and section by microtome (MICROM GmbH) at 5 μm and stained by hematoxylin and eosin. The stained tissues on the slides were mounted in Permount for analyzed under the microscope (Saraiva *et al*., 2015). The melanomacrophages centres (MMCs) were observed and count under light microscope (Olympus CX33) which 100X magnification and captured through microscope digital camera (Olympus EP50) by EP view software, three focal fields were randomly selected for count (Sagun *et al*., 2006).

Water quality parameters

The water quality parameters: pH value, DO, and temperature measured by pH meter (HI98128) and DO meter (YSI MODEL 57), respectively. Ammonia conducted by phenate method measured the absorbance at 630 nm by spectrophotometer (AQUA-VBC). Total reared water *A. hydrophila* coliform count using selective media modified Rimler-Shotts agar (RS media) to culture *A. hydrophila* in reared water, bacteria concentration using by direct count method (Shotts and Rimler, 1973). The water quality parameters were measured at different concentrations of extract to determine effect of MPE on water parameters changing.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) by Duncan's multiple range tests, significance was accepted at 95%. SPSS version 28 (Duncan, 1955) was used as software to analyzed.

Results

Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The Minimal Inhibition Concentration (MIC) test of MPE was performed by using different concentrations of MPE (0, 0.78, 1.56, 6.25, 12.5, 25, and 50 ppm) in NB and *A. hydrophila* concentration was 10^8 CFU mL^{-1}. NB absent turbidity will be regarded as MIC. Considering the present study, the NB did not show turbidity at 25 ppm and 50 ppm, therefore the MPE at 25 ppm was conducted to MIC (Table 1). For the Minimal Bactericidal Concentration (MBC). The MPE at 25 ppm and 50 ppm of MPE *A. hydrophila* did not growth on NA. Therefore, 25 ppm of MPE was conducted to MBC (Table 2).

Table 1. Turbidity of NB at different concentrations of MPE

	MPE concentration (ppm)							
	0	0.78	1.56	3.12	6.25	12.5	25	50
Turbidity	+	+	+	+	+	+	-	-

[1/] + mean NB present turbidity, [2/] - mean NB absent turbidity

Table 2. *A. hydrophila* growth on NA at different concentrations of MPE

	MPE concentration (ppm)							
	0	0.78	1.56	3.12	6.25	12.5	25	50
***A. hydrophila* growth**	+	+	+	+	+	+	-	-

[1/] + mean *A. hydrophila* growth on NA, [2/] - mean *A. hydrophila* did not growth on NA

Hematological parameters

Red blood cell (RBC) counted on the second day, the RBC of G40 was the highest among the other groups. After that the RBC showed slightly decreased. In addition, RBC of G40 and G60 were significantly increased on the sixth day compared to the control group. On the seventh day, all groups which received MPE had significantly increased of RBC (Figure 3A). On the contrast, the white blood cell (WBC) counted of G20, G40, and G60 increased on the third day after challenged (Figure 3B). Furthermore, the hemoglobin concentration (Hb) of group which received MPE increased after the second day. Except for G20, Hb was not significantly different from the control group on the sixth and seventh days (Figure 3C).

Water quality parameters

Ammonia concentration level in the control group was significantly higher compared to the other groups on every day of the experimental period, particularly the highest levels on the fourth day 0.06 ± 0.003 ppm. G60 had significantly lower ammonia levels than the other groups on everyday (Figure

4D). The temperature, DO and pH value were not significantly different (Figure 4A, 4B, 4C). Furthermore, total *A. hydrophila* coliform count from reared water on RS agar of G20, G40, and G60 were significantly lower than control group and no different between groups (Figure 5B).

Survival rate and clinical signs

Survival rate (SR) was observed after challenging fish with *A. hydrophila*. SR of challenged control group (C) started to decreased after the first day of challenged. The SR of G20 and G40 was significantly decreased on the third day after challenged. After the fourth day, the SR of G60 started to decrease. The SR of control group, G20, G40, and G60 at the end of the experimental was 20%, 45%, 65%, and 90%, respectively (Figure 5A). The clinical symptoms of died fish from the *A. hydrophila* challenged group were observed, the symptoms of fish found that the fish had hemorrhagic all along the body (Figure 1).

Figure 1. Clinical symptoms of died *L. calcarifer* after challenged with *A. hydrophila*. Showing the hemorrhagic by yellow arrow along the body

Histopathological of Lates calcarifer

Melanomacrophage centres (MMCs) were found during histological observation of kidney and liver from G20, G40, and G60 (Figure 2). G20 kidney had the highest MMCs count (14.89 ± 0.59 cell $(mm^2)^{-1}$). Meanwhile, G40 and G60 had MMCs that are not significantly different between group. Furthermore, MMCs were found in the livers of every challenged group which no different quantities (Table 3).

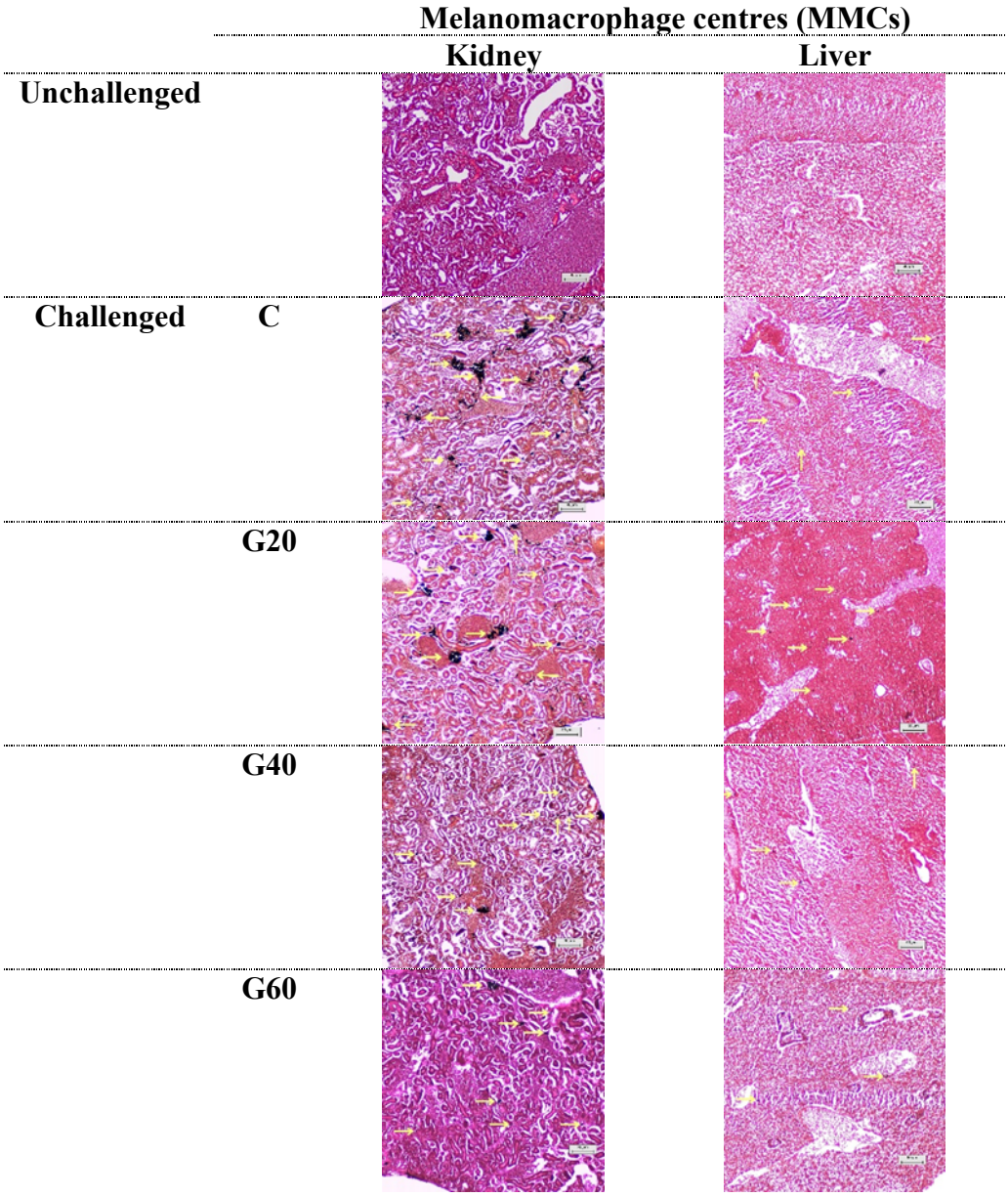

Figure 2. The histological observation of *L. calcarifer* after challenged with *A. hydrophila* and unchallenged. Haematoxylin-eosin staining. The magnification was 100X and scale bar was 90 µm. The yellow arrow showed melanomacrophage centres (MMCs) found in tissues

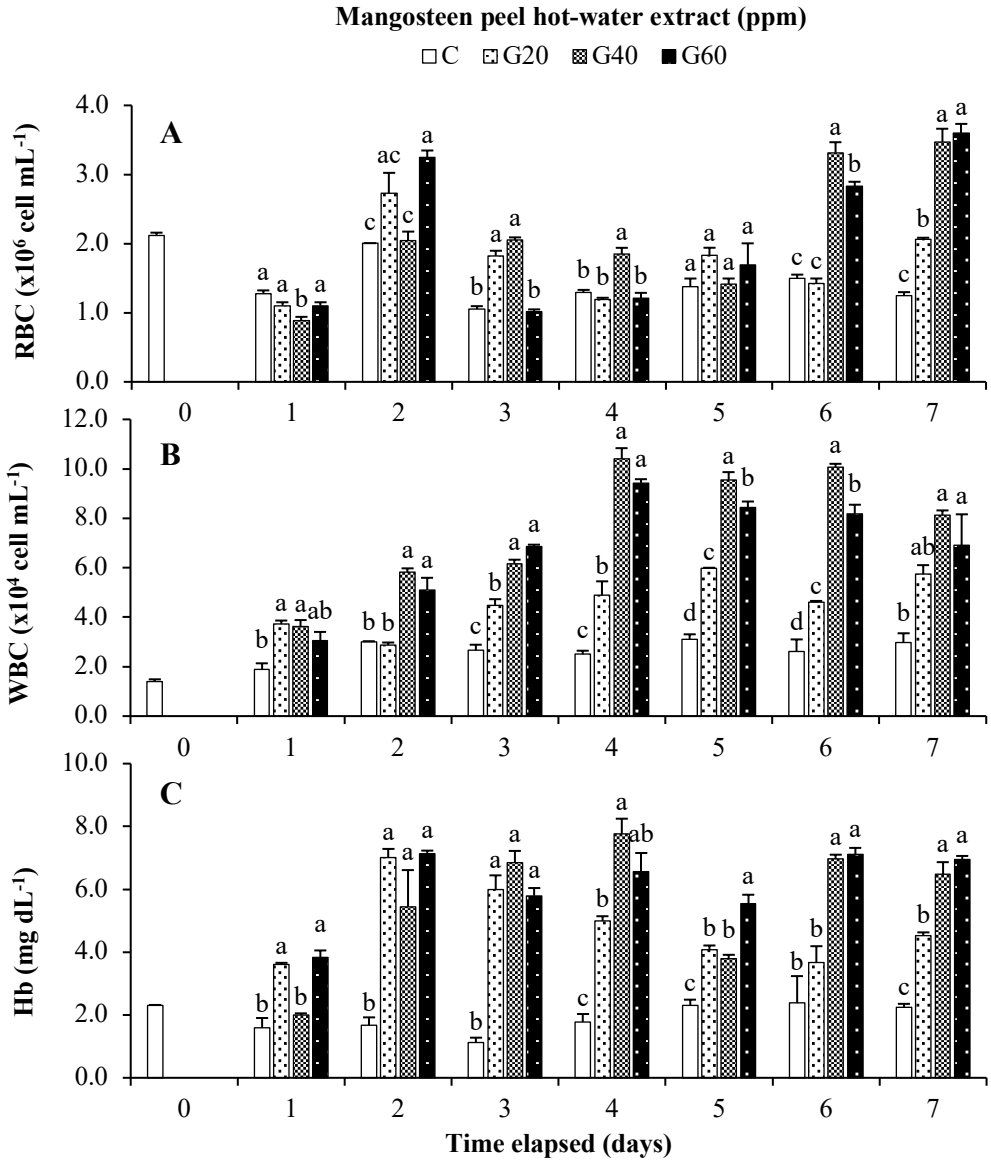

Figure 3. Red blood cell; RBC (A), White blood cell; WBC (B), and Hemoglobin concentration; Hb (C) of *Lates calcarifer* after challenged with *A. hydrophila* and reared in different concentration of MPE; 0ppm (C), 20ppm (G20), 40ppm (G40), and 60ppm (G60) for 7 days. The data represented (mean ± SE, n = 3 from 3 fish). Significant different ($P < 0.05$) indicated by the different letter

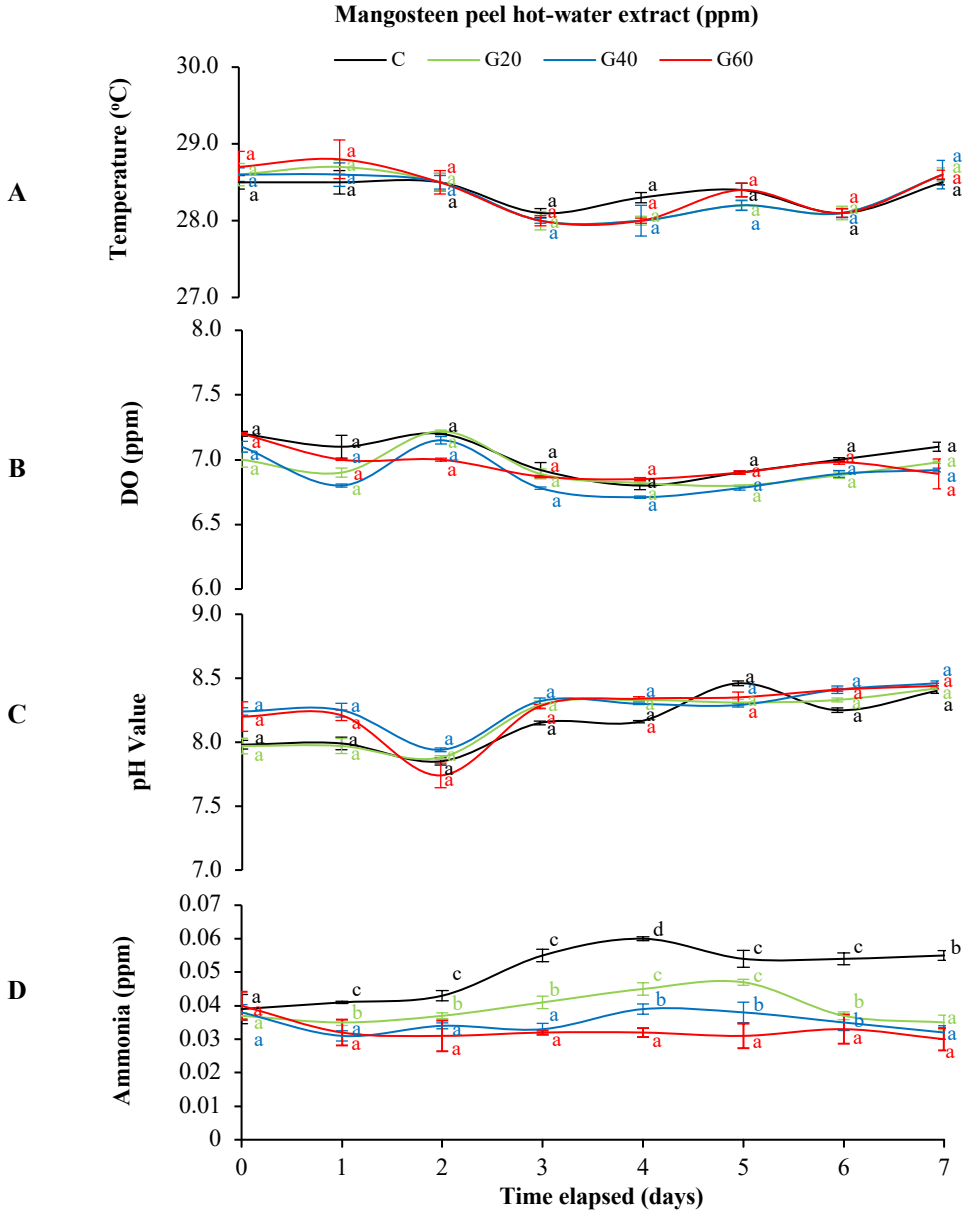

Figure 4. Temperature (A), Dissolved oxygen; DO (B), pH value (C), and ammonia concentration (D) in each day of experimental. Water samples were collected in different concentration of MPE; 0ppm (C), 20ppm (G20), 40ppm (G40), and 60ppm (G60) for 7 days. The data are represented (mean ± SE, n = 3 from 3 replications). Significant different ($P < 0.05$) indicated by the different letters

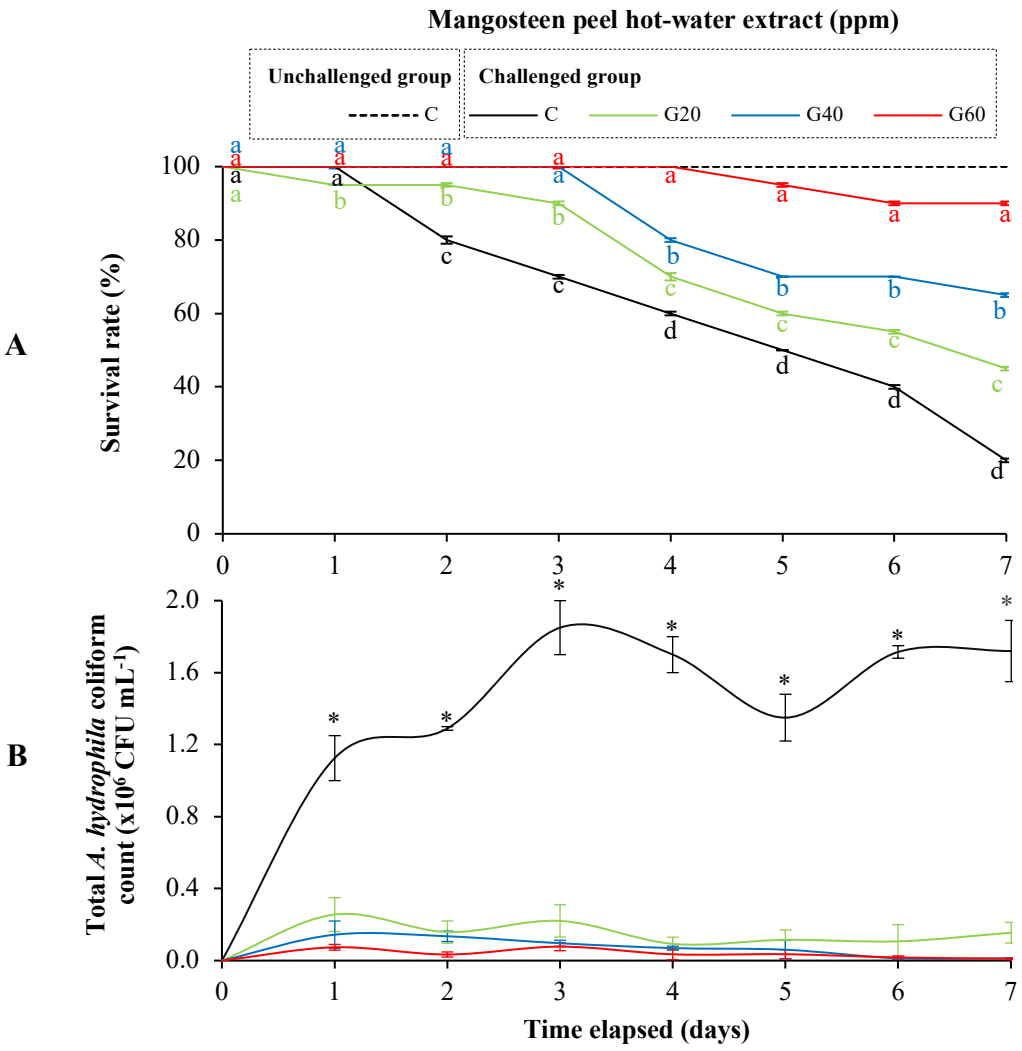

Figure 5. Survival rate (SR) of fish in different groups after 7 days (A). The data are represented (mean ± SE, n = 3, 10 fish per replicates). Significant different ($P < 0.05$) indicated by different letters. And total *A. hydrophila* coliform count from reared water in each day (B). Significant different ($P < 0.05$) indicated by asterisk. The data without asterisk mean no significantly. The data are represented (mean ± SE, n = 3 from 3 replicates). Which in different concentration of MPE; 0ppm (C), 20ppm (G20), 40ppm (G40), and 60ppm (G60) for 7 days. The unchallenged fish served as negative control

Table 3. Melanomacrophage centres (MMCs) count in kidney and liver in *L. calcarifer* after challenged with *A. hydrophila*

	MMCs count (cell $(mm^2)^{-1}$)	
	Kidney	Liver
Unchallenged	**-**	**-**
Challenged		
C	15.00 ± 0.76^b	3.78 ± 1.27^a
G20	14.89 ± 0.59^b	3.89 ± 1.36^a
G40	8.56 ± 0.88^a	3.44 ± 1.71^a
G60	7.56 ± 1.09^a	3.28 ± 0.32^a

[1/] **-** mean Non detected MMCs, The data are represented (mean ± SE, n = 3 from 3 focal areas observation). Significant different ($P < 0.05$) was indicated by the different letters.

Discussion

The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of mangosteen peel extract by hot water (MPE) on *Aeromonas hydrophila* (10^8 CFU mL^{-1}) were 25 ppm, in contrast to the study of mangosteen leaf extract by alcohol (Deangroj *et al*., 2022). Mangosteen leaf extract MIC was 1,563 ppm. According to Pinto *et al*. (2005), the biochemical isolated from mangosteen peel contains more biochemical compounds than those extracted from mangosteen leaves, bark, or other parts of the mangosteen tree. Furthermore, the immunomodulator was the one of properties which biochemical from mangosteen peel extract could enhance. Therefore, the mangosteen peel was chosen for usage. In study to use mangosteen peel extract to against fish bacterium *Streptococcus agalactiae* by different solvents use in extraction, found that the MIC of mangosteen peel extract from distilled water at 25 ^oC for 24 hr was 1,170 µL mL^{-1} (Pachanawan and Ngamsnae, 2010). Despite of the similar solvent was used in this study the MIC of mangosteen peel extract by boiled condition was different. Applying the extraction procedure with hot water was the simplest and most affordable method. Furthermore, an *In vitro* study using mangosteen peel extract by ethanol for antibacterial research was conducted found that mangosteen peel extract is effective against gram-negative bacteria (Indrianingsih *et al*., 2019).

The red blood cell (RBC) and hemoglobin concentration (Hb) of hybrid catfish (*Clarias macrocephalus* x *C. gariepinus*) infected by *A. hydrophila* were not significantly different from the control group while the white blood cell

(WBC) of infected fish increased (Wangkahart, 2018). The research on neutrophil prevention in *A. veronii*: because of the immunological response, the WBC was the key factor in protecting the host from bacterial infection by increasing when the pathogens exposed (Havixbeck *et al*., 2017). The study effect of *A. hydrophila* infection in goldfish (*Carassius auratus*), the level of RBC and Hb of fish were decreased after infection because RBC was destroyed by bacterial leading to anemia and WBC increased (Harikrishnan *et al*., 2010). Mangosteen shoot extract enhanced the hematological parameters of African catfish (*C. gariepinus*) fingerlings by increasing RBC and WBC (Soosean *et al*., 2010). According to present study the hematological parameters, RBC in the groups which received MPE 40ppm (G40) and 60ppm (G60) were slightly decreased on the second day after infected by *A. hydrophila* then significantly increased on the sixth and seventh day. Which was consistent with Hb of the MPE received group being higher than the control group. Because the biochemicals in MPE contain immunostimulant and antibacterial properties, the MPE received group had higher RBC and Hb than the control group. These results suggest that the MPE was able to recover the fish from infection by enhancing hematological parameters. After the second day of infection, all groups which received MPE had an increase in WBC which started to decrease on sixth and seventh day after infection, whereas RBC increased on the same day. These reversible alterations indicated that the fish had recovered from infection. Following by the study of Lili *et al*. (2019), on the third day, the mangosteen rind solution able to recovery the *A. hydrophila* infected tilapia (*Oreochromis niloticus*) juveniles. Observed by clinical symptoms and fish response. The fish were received mangosteen rind solution showed the response to feed on the third day after infection. In addition, the survival rate of the control group and group received 20 ppm of MPE (G20) was slightly lower on the third day as a result of a decreased in RBC. The *A. hydrophila* was certainly causing damage to the fish on the third day after infection. According to the studies, seabass (*Lates calcarifer*) infected with *A. hydrophila* had a higher cumulative mortality on the third day after infection (Ali *et al*., 2017). Betta fish (*Betta* sp.) infected with *A. hydrophila* had a lower survival rate on the second day after infection (Nugroho *et al*., 2017). The group received 40 ppm (G40) and 60 ppm (G60) of MPE had higher survival rate while group received 20 ppm of MPE (G20) had a lower rate. Because MPE at 20 ppm was lower than the MBC of MPE. According to the results, the application of MPE enhanced the survival rate of fish exposed to *A. hydrophila*. Furthermore, the observation from died fish showed hemorrhagic along the body. The observation by Deen *et al*. (2014) and

El-Sherbeny *et al*. (2022). Similar to tilapia (*O. niloticus*) infected with *A. hydrophila* displayed hemorrhagic along the body.

The histological observation of the melanomacrophage centres (MMCs) were found in kidney and liver tissues while the kidney has a higher amount of MMCs than the liver. MMCs are formed by the aggregation of macrophages found in the kidney, spleen and occasionally the liver. MMCs occur in fish, amphibian and reptile organs (Steinel and Bolnick, 2017). MMCs were often use as biomarkers when fish were exposed to stress, pathogens or environmental changes (Dang *et al*., 2019). Another study revealed MMCs found in the kidney tissue of tilapia (*O. mossambicus*) infected with *A. hydrophila* (Azad *et al*., 2001). MMCs were found in the kidneys of seabass (*L. calcarifer*) reared in freshwater and infected with *S. iniae* (Kayansamruaj *et al*., 2015). According to present study, MMCs found in the kidney of group which received 20 ppm of MPE (G20) were the most abundant and appeared to span a larger area than the other groups because of the lower quantity. Application MPE at 40 ppm and 60 ppm have the potential to reduce the virulence of *A. hydrophila* infection.

The water quality parameters of pH value, DO, and temperature were not significantly different between every group. According to the study by Lemarie *et al*. (2004). High ammonia concentrations in European seabass (*Dicentrarchus labrax*) reared water cause mortality, weight loss, and growth stagnation. The present study, concentration of ammonia in groups which received MPE was lower than in the control group. According to Pimpimol *et al*. (2020), the application of tannin extract from mangosteen peel was effective to removing the ammonia in water. Furthermore, tannin extracted from the heaviest weight of mangosteen peel has the highest tannin content. In this study, group received 60 ppm of MPE (G60) had the lowest ammonia concentration, followed by group which received 40 ppm (G40) and 20 ppm (G20) of MPE. The results were consistent with earlier study, G60 being expected to have the highest tannin concentration. As a result, application 60 ppm of MPE had the efficacy in reducing the ammonia concentration in rearing water. The rearing water *A. hydrophila* coliform count, the tank applicated MPE showed a decrease of *A. hydrophila*, which was investigated using Rimler-Shotts agar to identify (Rahman *et al*., 2021). The decreasing of *A. hydrophila* colonies in reared water was an indicator to antibacterial properties of MPE by lowered bacteria colonies.

In conclusion, MPE is shown to be effective against *A. hydrophila*. In terms of culture, the application of MPE is recommended for improving fish hematological parameters and preventing bacteria infection. Furthermore, the application of MPE able to improve water quality in the culture system.

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