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Citrus microcarpa extract as bio-immunostimulator for Edwardsiellosis in red hybrid tilapia culture

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Abstract

This paper described the application of *Citrus microcarpa* extract as bio-immunostimulator in red hybrid tilapia against Edwardsiellosis infection. Edwardsiellosis due to *Edwardsiella tarda* is one of the well-known bacterial diseases in aquaculture which leads to significant economic losses. The increasing antibiotic resistant cases among pathogenic bacteria led to many commercial antibiotics no longer effective in controlling bacterial diseases in aquaculture. Hence, in the present study was carried out to evaluate potential of *C. microcarpa* extract as immunostimulator against Edwardsiellosis infection in red hybrid tilapia. Comparison in terms of cumulative mortalities and antibody response against *E. tarda* among group of fish received *C. microcarpa* extract at different concentrations (CM1-1, 1 g kg^{-1} of fish; CM-2, 2 g kg^{-1} of fish and CM-4, 4 g kg^{-1} of fish) and group of fish received no medicated commercial feed (control) was carried out in the present study. Enzyme linked immunosorbent assay (ELISA) was used to monitor antibody response of fish that received medicated feed. The results of the present study showed that the values of antibody response against *E. tarda* of fish after seven days received *C. microcarpa* extract (CM-1, 0.113 ± 0.02 OD; CM-2, 0.14 ± 0.02 OD; CM-4, 0.173 ± 0.03 OD) were significantly higher ($P < 0.05$) compared to fish from group of control (0.0 OD). Whereas cumulative mortality of fish from group of control (53.3 ± 11.5 %) was significantly higher ($P < 0.05$) compared to fish from all of groups received *C. microcarpa* extract (CM-1, 13.3 ± 5.8 %; CM-2, 13.3 ± 5.8 % and CM-4, 6.7 ± 5.8 %). The results indicated the potential of *C. microcarpa* extract as immunostimulator in finfish culture.

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1. INTRODUCTION

Edwardsiellosis due to *Edwardsiella tarda* was reported caused huge economic losses in important freshwater and marine fish culture (Thune et al., 1993; Plumb, 1999). This bacterium can be found widely in aquatic environment. *E. tarda* has been successfully isolated from various aquatic animals, including the Asian clam, *Corbicula fluminea* (Lee et al., 2013), giant freshwater prawn, *Macrobrachium rosenbergii* (Lee et al., 2009a), American bullfrog, *Rana catesbeiana* (Lee et al., 2009b), Asian seabass, *Lates calcarifer* (Lee et al., 2010a), silver catfish, *Pangasius sutchi*, red hybrid tilapia, *Tilapia* sp. (Lee et al., 2010b) and ornamental fish (Najiah et al., 2008),. Traditionally, antibiotic will be used to against this bacterial disease in fish. However, antibiotic resistance cases of *E. tarda* have been widely recorded in worldwide aquaculture over the years (Walman and Shotts, 1986; Aoki and Takahashi, 1987; Aoki et al., 1989; Lee et al., 2009; Park et al., 2017). *E. tarda*. Lee and Najiah (2008) reported that *Allium sativum* extract can be used to inhibit the growth of *E. tarda*. Recently, *C. microcarpa* extract possess antimicrobial property against various types of pathogenic bacteria including *E. tarda* isolated from various types of aquatic animals (Lee et al., 2008; Lee and Najiah, 2009) and citric acid was the major bioactive compound in *C. microcarpa* extracts which responsible to the antimicrobial property of this plant extract (Lee and Najiah, 2009). Therefore, it is a must to further the study on the effectiveness of *C. microcarpa* in controlling *E. tarda* infected in fish.

2. MATERIALS AND METHODS

2.1. Bacteria culture

Edwardsiella tarda was isolated from diseased red hybrid tilapia farmed in Kelantan, Malaysia. The bacterial isolate was cultured using brain heart infusion broth (Oxoid, England) for 18 h at room temperature. Bacterial pellet was harvested by centrifugation at 13,500

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rpm for 10 min. The harvested bacterial pellet was washed twice using physiological saline and the concentration of the bacterial isolate was adjusted to 10^9 colony forming unit (CFU mL^{-1}) to be used in challenge test by intraperitoneal injection of 100 µL inoculum to the fish samples.

2.2. Crude extract preparation

Citrus microcarpa fruits bought from the local market were subjected to ultraviolet sterilization for 30 min prior to extraction process according to Daud et al. (2005) and Yano et al. (2006) with some modifications. Crude extract was prepared by cutting the fruit into half and finely blend with sterile distilled water, followed by filtration using Whatman filter paper No 1. The extract was then stored at 4 ℃ for further use.

2.3. Feed preparation

Medicated feed was prepared by spray-coating the commercial fish pellet (Star Feedmills, Malaysia) with different concentrations of *C. microcarpa* fruit extract; 1 g kg^{-1} of fish (CM-1), 2 g kg^{-1} of fish (CM-2) and 4 g kg^{-1} of fish (CM-4). Subsequently, the coated pellets were oven dried at 30 ℃ for 24 h and stored at 4 ℃ until further use.

2.4. Experimental design

A total of 150 farmed red hybrid tilapia fries with the average weight (10±0.5 g) was acclimated at (aquaculture hatchery, Universiti Malaysia Kelantan, Jeli) for 4 weeks before the challenge test. Fish samples were divided into 15 groups, where each group consisted of 10 fish was maintained in a 20 L aquarium. Nine groups of fish were used in the treatment with *C. microcarpa* fruit extract at different concentrations; 1 g kg^{-1} of fish (CM-1), 2 g kg^{-1} of fish (CM-2) and 4 g kg^{-1} of fish (CM-4), and another 6 groups of fish served as the control. Triplicate groups of fish were used for each treatment, positive control and negative control. Treatments CM-1, CM-2 and CM-4 coated onto commercial fish pellets were fed to the respective group of fish at 2% body weight of fish per day for a week, before the bacterial challenge and continued for another four weeks. For the bacterial challenge, fish under treatments were subjected to intraperitoneal injection with 100 µL inoculum of *E. tarda*. Cumulative mortality of the infected fish was observed and recorded up to four weeks. Fish from each treatment was randomly sampled for indirect enzyme linked immunosorbent assay (ELISA) on weekly basis after the challenge.

2.5. Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was carried out as described by Shelby et al. (2002) with some modifications. Fish were bled from the caudal vein and the blood was collected into micro centrifuge tube. The blood was then allowed to clot for 1 h at 25 ℃. The fish serum was harvested through centrifugation at 300 *g* and stored at –80 ℃ for further use. Edwardsiellosis antigen was prepared by dilution of *E. tarda* whole cell with sodium carbonate buffer to 500 µg mL^{-1}. A hundred microliter of edwardsiellosis antigen was added into each well of a microtitre plate for 1 h at 25 ℃. The wells were then blocked with 3% bovine serum albumin (Sigma, USA) for 1 h at 25 ℃. After the incubation period, the wells were washed five times with PBS plus Tween-20 (PBS-T). A hundred microliter of a serum sample (1 µL of serum diluted in 999 µL of PBS-T) was added to three replicate wells of the plate followed by 30 min incubation at 25 ℃. The wells were then washed three times with PBS-T. After washing, 100 µL of goat anti-tilapia immunoglobulin serum (diluted 1:5000 in PBS-T) was added into the wells followed by 30 min incubation at 25 ℃. After three times repeated washing with PBS-T, 100 µL of rabbit anti-goat peroxidase conjugate (diluted 1: 5000 PBS-T) was added into the wells. Finally, the wells were washed again with PBS-T followed by the addition of 100 µL of *o*-phenylenediamine in urea-peroxide buffer into each well. The ELISA reaction was stopped at 15 min by adding 50 µL of 3 M H_2SO_4. The optical densities (OD) of the reactions were read with a microplate reader (Bio Rad, USA) at 490 nm. Negative controls consisted of wells coated with antigen and without sample serum, and wells with no antigen and a serum sample. The control reactions gave an OD of 0.04 or less.

2.6. Statistical analysis

Fish cumulative mortality and ELISA values were analysed with one-way ANOVA using Tukey's post- hoc test at 5% of significant level ($p < 0.05$).

3. RESULTS AND DISCUSSION

In the first week of experiment, significant differences ($p < 0.05$) in fish antibody titres measured by ELISA were found in fish that received *C. microcarpa* extract (CM-1, 0.113 ± 0.02 OD; CM-2, 0.14 ± 0.02 OD; CM-4, 0.173 ± 0.03 OD) and fish from control group (0.0 OD). However, no significant differences ($p > 0.05$) were observed in fish antibody titres from all groups of fish at the end of experiment. The percentage of cumulative mortality of red hybrid tilapia was significantly different ($p < 0.05$) between fish from group of control (53.3 ± 11.5 %) and fish from all the groups received *C. microcarpa* extract (CM-1, 13.3 ± 5.8 %; CM-2, 13.3 ± 5.8 %; CM-4, 6.7 ± 5.8 %).

To our knowledge this study was the first report on the usefulness of *C. microcarpa* as stimulator of fish immune system against edwardsiellosis due to *E. tarda*. Traditionally, fish farmer using antibiotic in controling fish disease in aquaculture. For instance, Stock and

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Wiedemann (2001) revealed the susceptibility of 42 bacterial isolates of *E. tarda* against 71 types of antibiotics as well as Clark et al. (1991) also investigate the sensitiveness of *E. tarda* to 22 types of antibiotics. However, fish farmers were not encouraged to use antibiotics in aquaculture especially in food fish farming due to public health concern and environmental hazard. The application of antibiotic in aquaculture may lead to the contamination of antibiotic residue in the aquaculture product that will pose a threat to human health if not managed well. Some worst scenario occurred when the contaminated aquaculture product is rejected by many countries. Hence, it is a must to find the alternative way to overcome the arise problem. Therefore, alternative antimicrobial agents such as plant extracts were used for controlling disease in fish farm. Till present, a lot of studies have been carried out to reveal the potential natural antimicrobial agent in controlling edwardsiellosis due to *E. tarda*. For instance, Lee et al. (2009) found that the essential oil of *Syzygium aromaticum* flower bud (clove) was able to inhibit the growth of *E. tarda*. Other plant extracts that reported possess inhibitory activities against *E. tarda* were *Andrographis paniculata* (Lee et .al 2011a), *Michelia champaca* seed and flower (Lee et al. 2011b), *Peperomia pellucida* leaf (Lee et al., 2011), *Phyllanthus urinaria* leaf (Lee et al., 2012) and citronella, *Cymbopogon nardus*, essential oil (Lee and Wendy, 2013). Recently, Lee et al. (2009) revealed that *C. microcarpa* extract possess antimicrobial property against various types of bacteria isolated from aquaculture sites and 2-Hydroxypropane-1,2,3-Tricarboxylic Acid was responsible to the antimicrobial activity of *C. microcarpa*. However, till to date, the effectiveness of antimicrobial agents in controlling edwardsiellosis in fish were still lacking in the literature. Therefore, the result of the present study may useful for fish farmer in selecting natural antimicrobial agents for fish health management purpose.

4. CONCLUSION

Citrus microcarpa was found can be used as antimicrobial agent in controlling Edwardsiellosis due to *Edwardsiella tarda* in red hybrid tilapia culture, farming.

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