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Citrus microcarpa Extract as Immunostimulator in Controlling Edwardsiellosis in African Catfish Culture

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Abstract

This study was described Citrus microcarpa extract as an antimicrobial agent in controlling edwardsiellosis in African catfish culture. Edwardsiellosis due to Edwardsiella tarda was recognized as a problem in African catfish culture and may lead to mass mortality of the cultured fish. Many antibiotics were no longer effective to control this bacterial disease. Therefore, this study was carried out to investigate the potential of C. microcarpa extract to overcome this bacterial disease problem. The experimental fish were fed with medicated feed at three different concentrations (1 g kg-1; CM-1, 2 g kg-1; CM-2 and 4 g kg-1 of fish) of C. microcarpa extract for one week before they were intraperitoneally exposed to E. tarda. Enzyme linked immunosorbent assay (ELISA) was carried out to determine the value of antibody response to E. tarda in fish from group of fish that received medicated fish and the percentage of total cumulative mortality of the experimental fish were observed at the end of the experiment. The results showed that the value of antibody response to E. tarda in fish from group of fish which received medicated feed (CM-1, 0.148 ± 0.017 OD; CM-2, 0.143 ± 0.006 OD; CM-4, 0.163 ± 0.015 OD) were found significantly higher ($P < 0.05$) compared to fish did not received medicated fish (0.00 OD). Whereas, percentage cumulative mortality of fish from all groups of fish received medicated feed (CM-1, 20.0 ± 10.0 %; CM-2, 16.7 ± 5.8 %; CM-4, 16.7 ± 5.8 %) were found significantly lower ($P < 0.05$) compared to group of fish did not received medicated feed (56.7 ± 5.8 %). The findings of the present study indicated the huge potential of C. microcarpa extract as natural antimicrobial agent for aquaculture use.

1. Introduction

Disease is a major problem in aquaculture and bacterial disease was recognized as a common pathogen which is significant setback for successful aquaculture not only in Malaysia but also worldwide. One of the well known bacterial diseases was edwardsiellosis. Edwardsiellosis due to Edwardsiella tarda was reported infected various species of fish such as carp, tilapia, eel, catfish, mullet, salmon, trout and flounder (Mohanty and Sahoo, 2007). In

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Malaysia, this bacterium was reported successfully isolated from Asian swamp eel (Najiah et al., 2006), red hybrid tilapia, snakeskin gourami (Lee and Najiah, 2008), ornamental fish (Najiah et al., 2008), giant freshwater prawn (Lee et al., 2009a), American bullfrog (Lee et al., 2009b) and African catfish (Najiah et al., 2009). Although no exact figure of loss was recorded but E. tarda posed a significant problem in Malaysia aquaculture can not be denied. Furthermore, in Malaysia, several cases of mass mortality of African catfish were occurred due to this bacterium during the operation of hatchery and growth out pond as well. The bacterium was also reported infected in human, reptiles, bird and others mammals which are well documented in literature. In spite of these facts, there is no successful established treatment or prevention against this bacterial disease. In this paper, we revealed the potential C. microcarpa extract in controlling E. tarda in African catfish.

2. Materials and methods

2.1 Bacterial isolate

Edwardsiella tarda isolated from diseased African catfish (C. gariepinus) at commercial farms in Kelantan, Malaysia was used in the experiment. Phenotypic, genotypic and whole cell protein profiles of this bacterial strain were previously described by Lee and Najiah (2008). A preliminary study was conducted showing that the minimum inhibitory concentration (MIC) value of C. microcarpa extract against the present bacterial strain C3 was 4 g L-1. The bacterial isolate were cultured using brain heart infusion broth (Oxoid, England) for 18 h at room temperature. The bacterial pellet was harvested by centrifugation at 13,500 rpm for 10 min. The harvested bacterial pellet was washed twice using physiological saline and the concentration of the bacterial isolate was adjusted to 109 colony forming unit (cfu) mL-1 for challenged by intaperitoneal (i.p) injection of 100 µL of each inoculum, at a dose causing 50 % mortality (LD50).

2.2 Plant extraction

C. microcarpa extract was prepared immediately after bought from local market. The plants were subjected to ultraviolet for 30 min. C. microcarpa was extracted according to Daud et al. (2005) and Yano et al. (2006) with some modifications. Briefly, it was cut and finely blended. The extract was then stored at 4 oC for further use.

2.3 Medicated feed

The fish pellets (Star, Malaysia) were purchased commercially before they were mixed with crude extract of C. microcarpa. Medicated feed was consisted of three different concentrations (1 g kg-1, 2 g kg-1 and 4 g kg-1 of fish) of C. microcarpa extract. The extract was coated with fish pellet at a desired concentration and oven dried at 30 oC for 24 h. The prepared fish pellet was then kept at 4 oC for further use.

2.4 Experiment design

The antimicrobial agent efficacy test was carried out to determine the effectiveness of C. microcarpa extract in preventing and controlling Edwardsiellosis in African catfish due to E. tarda. A total of 15 groups of fish, where each group contain 10 fish were maintained in 20 L aquaria. Six groups of fish were used as control which each three groups served as negative and positive control. 9 groups of fish were used as treatment for three different concentration of C. microcarpa 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) which each treatment contained a triplicate. The experimental fish were given medicated fish pellet at 2 % body weight of fish per day for one week before the fish were exposed to E. tarda by intraperitoneal injection. The mortality of the infected fish was observed and recorded for four weeks for. Simultaneously, the medicated and unmedicated fish pellet was continuously given to the fish for four weeks. Fish from each treatment was randomly sampled for enzyme linked immunosorbent assay (ELISA) for every week.

2.5 Indirect enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) was carried out as described by Shelby et al. (2002) with some modification. Briefly, fish were bled

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from the caudal vein and the blood was collected into micro centrifuged tube. The blood was then allowed to clot for 1 h at 25 oC. The fish serum was harvested through centrifuged at 300 g and stored at – 80 oC for further use. The edwardsiellosis antigen was prepared by diluted whole cell of E. tarda with carbonate buffer to 500 µg mL-1. A hundred µL of edwardsiellosis antigen was added into each well of microtitre plate for 1 h at 25 oC. The wells were then blocked with 3 % bovine serum albumin (Sigma, USA) for 1 h at 25 oC. After the incubation period, the wells were washing 5 times with PBS plus teewn-20 (PBS-T). A hundred µL of a serum sample (1 µL of serum diluted in 999 µL of PBS-T) was added to three replicate wells of plate followed by 30 min incubation at 25 oC. The wells were then washed 3 times with PBS-T. After washing, a hundred 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 oC. After 3 times washing again with PBS-T, a hundred 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 a hundred µL of o-phenylenediamine in urea-peroxide buffer was added to each well. The elisa reaction was stopped at 15 min by adding 50 µL of 3 M H2SO4. The optical densities (O.D) of the reactions were read with microplate reader (Bio Rad, USA) at 490 nm. Negative controls consisted with wells coated with antigen and no 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

Statistical differences between mortality and ELISA values were analyzed with one-way analysis of variance using Tukey post hoc multiple comparison tests at 5% of significant level.

3. Results

In the first seven days of the experiment, the value of antibody response to E. tarda in fish from group of fish that received medicated fish (CM-1, 0.148 ± 0.017 OD; CM-2, 0.143 ± 0.006 OD; CM-4, 0.163 ± 0.015 OD) were found significantly higher ($P < 0.05$) compared to fish did not received medicated fish (0.00 OD) (Table 1). On the other hand, in the end of the experiment, percentage cumulative mortality of fish from all groups of fish received medicated feed (CM-1, 20.0 ± 10.0 %; CM-2, 16.7 ± 5.8 %; CM-4, 16.7 ± 5.8 %) were found significantly lower ($P < 0.05$) compared to group of fish did not received medicated feed (56.7 ± 5.8 %) (Table 2).

Table 1. Antibody response value of fish to Edwardsiella tarda

Treatment	Antibody value (OD)
Control	0.00
CM-1	0.148 ± 0.017*
CM-2	0.143 ± 0.006*
CM-4	0.163 ± 0.015*

* $P < 0.05$ significantly different compared to control treatment

Table 2. Cumulative mortality of treated fish to Edwardsiella tarda infection

Treatment	Cumulative mortality (%)
Control	56.7 ± 5.8
CM-1	20.0 ± 10.0*
CM-2	16.7 ± 5.8 *
CM-4	16.7 ± 5.8*

* $P < 0.05$ significantly different compared to control treatment

4. Discussion

Recently, antibiotic resistance case among pathogenic bacteria was reported increase rapidly. Many commercial antibiotics were found no longer effective to control bacteria disease such as edwardsiellosis in African catfish culture. Furthermore, due to the concern of environmental hazard and public health, many countries was discouraged their fish farmer to apply antibiotic in aquaculture. Therefore, it is important for scientists from all over the world to find the alternative source instead of antibiotic as antimicrobial agent for aquaculture use. The ELISA results of the present study indicated that *C. microcarpa* extract can increase immune system of African catfish. Subsequently, African catfish which was fed with *C.*

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microcarpa extract was found resistant to edwardsiellosis due to *E. tarda*. To our knowledge, this is first report on the effectiveness of *C. microcarpa* in controlling edwardsiellosis in African catfish. At present, many studies were reported antimicrobial property of *C. microcarpa*. For instance, Lee et al. (2008) claimed that *C. microcarpa* can inhibit the growth of bacteria from aquacultures sites. Furthermore, Lee and Najiah (2008) revealed that citric acid was responsible to the antimicrobial activity of *C. microcarpa* extract. Up to date, there is no literature on the trial on ***C. microcarpa*** extract as antimicrobial agent in aquaculture. However, four types of citrus fruit (grapefruit; *Citrus paradisi*, mandarin orange; *C. reticulata* **B**lanco, bergamot; *C. aurantium* L. ssp. Bergamia and sweet orange; *C. sinensis*) were found can be used as growth promoter in chicken and swine farming in which survival rate, weight gain and food conversion rate of chicken and swine that fed with these citrus fruits were found significantly higher than those fed with commercial antibiotics such as colistin, virginiamycin, flavomycin and avilamycin (Mellor, 2002). In the present study, *C. microcarpa* extract was found useful in overcome edwardsiellosis problem in African catfish culture. Further study on the effectiveness of this plant in controlling other fish diseases should be carried out before the conclusion of this plant extract may alternate antibiotic as antimicrobial agent for aquaculture use can be made. For this moment, the field test of this plant extract is still undergone.

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