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Virulence of *Aeromonas hydrophila* in Siamese fighting fish (*Betta splendens*) and the bacterium susceptibility to some herbal plants

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Abstract

Aeromonas hydrophila was isolated from diseased Siamese fighting fish *Betta splendens* Regans. The virulence of *A. hydrophiala* to Siamese fighting fish was conducted by intraperitoneal injection of two doses of bacterial suspensions with 7.5×10^7 and 7.5×10^5 cfu per 0.05 ml. The cumulative mortality was observed in high and low dose groups which showed 98.33 and 20 percent, respectively. Moreover, five medicinal plants extracts namely *Centella asiatica, Morinda citrifolia, Melissa officinalis, Piper sarmentosum* and *Terminalia catappa* were determined for antimicrobial activities by broth dilution method. *Terminalia catappa* extract showed highest antimicrobial effect of MIC and MBC (25 and 12.5 mg mL^{-1}), followed by *M. officinalis* and *P. sarmentosum* with the same values of MIC and MBC (12.5 and 25 mg mL^{-1}). Finally, *Centella asiatica* and *Morinda citrifolia* were similarly MIC and MBC (50 and 100 mg mL^{-1}). It was concluded that five medicinal plants can use to inhibit and kill *A. hydrophila* in fish.

Keywords: *Aeromonas hydrophila*, *Betta splendens*, Antibacterial activity, Medicinal plants

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Introduction

Siamese fighting fish is an economically major species of ornamental fish in Thailand. As an ornamental fish, the males have more attractive colors and fins (Amiri and Shaheen, 2012). Bacterial diseases are the most common infectious problem of ornamental fish, and the most bacterial infections are caused by gram-negative organisms including *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium* (*Flexibacter*), *Pseudomonas* and *Vibrio* (Lewbart, 2008; Buller, 2004) genera. Especially, *Aeromonas* species has been documented as a pathogenic organism in ornamental fish (Jagoda *et al*., 2014). Outbreaks of motile aeromonad septicaemia usually occur whether fish are immune compromised due to unpleasant environment or predisposing factors leading to stresses such as temperature, overcrowding, organic pollution and hypoxia (Anusha *et al*. 2014). Diagnosis of bacterial diseases of fish required observation of common clinical signs associated with the disease, isolation and identification of the causative bacterial organisms and confirmation with animal inoculation is important to fish culturist and fisheries managers (Abowei and Briyai, 2011). The degree of virulence of its bacterium therefore is importantly studied in the aquaculture filed. Moreover, the most common approach to eliminate bacterial diseases is using antibiotics (Yanong, 2006; Rattanachaikunsopon and Phumkhachorn, 2009). Therefore, mistreatment of any antibiotics can leads to the founding of resistant bacteria and accumulation of antibiotic residues in the environment. The alternative treatment by using some medicinal plants has been investigated for pathogenic bacteria (Harikrishnan *et al*., 2011; Anusha *et al*., 2014; Prasad , 2014). The aim of the present study was to study the pathogenesis of *A. hydrophila* against Siamese fighting fish and to study the antibacterial activity of some medicinal plants against mentioned bacteria.

Materials and methods

Fish

Ninety-healthy Siamese fighting fish (average 3.67 g in body weight and 9.72 cm in body length) were used in this study. The experiments were carried out at the Faculty of Agricultural Technology, Rajabhat Maha Sarakham University in April 2014.

Preparation of the inocula

A. hydrophila RMU 5603 isolated from diseased Siamese fighting fish in 2013 was used in challenges. Concentrations of bacterial suspension were adjusted to $7.5x10^7$ and $7.5x10^5$ cfu per 0.05 ml by compared to the 0.5 McFarland standards.

Experimental infection

Ninety fish (30 fish group^{-1}) without clinical signs were used for experiment infection. Two groups of fish were challenged with high and low doses of inocula, respectively. Each fish was intraperitoneally injected with 0.05 mL bacterial suspension. The fish in the third group was injected with sterile

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normal saline served as a negative control group. All experiment groups were maintained in 1 L of individual aquarium per fish. Mortalities were recorded every 12 h for 2 weeks post injection with bacterial suspension. Both dead and survived fishes were re-isolated from the kidney for evaluated the etiological evidence of fish. Bacteria were re-isolated and identified by biochemical tests (Buchanan and Gibbon, 1974) and API 20E (bioMerieux, France).

Herbal extracts for in vitro antibacterial activity against A. hydrophila

Five herbal extracts including *Centella asiatica, Morinda citrifolia, Melissa officinalis*, *Piper sarmentosum* and *Terminalia catappa* were carried out according to Ponnusamy *et al*. (2010) with slight modification. Briefly, 50 g of each plant powder and 200 mL of 95% ethanol. Then, the aqueous extracts were filtered and concentrated in vacuum at 40 ◦C using rotary evaporator. These extracts were dissolved in dimethyl sulphoxide to make the final concentrations.

Determination of minimum inhibitory concentration

In order to determining minimum inhibitory concentration (MIC), a serial dilution of each extract (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 mg L^{-1} were prepared in sterile 96-well microtiter plate. Then, the bacterial suspensions were added to each well and incubated at 30 °C for 18-24 hours according to Prasad (2014) with some modifications. The experiments were conducted in triplicate. The MIC was observed in the minimum concentration of each plant extract that could be inhibited the growth of bacteria.

Determination of minimum bactericidal concentration

Fifty microliter of each clear well were inoculated into Muller Hinton Agar and incubated at 30 °C for 18-24 hours. The experiments were done in triplicate. The MBC was observed in the minimum concentration of each plant extract that could be killed the growth of bacteria.

Results

Mortality

The cumulative mortality of Siamese fighting fish challenged with *A. hydrophila* at 14 days post injection was 98.33 and 20 percent in high and low doses of inocula, respectively. In addition, the mortality in control group was 0 %. The abnormal symptoms were hemorrhage in the vent, protruding scales, exophthalmia and abdomen distention in experimental fish (Fig. 1). The mortality records were corresponded with re-isolation from the kidney of moribund fishes. Thus it seems that *A. hydrophila* was a pathogenic bacteria base on the results of mortality.

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Figure 1: A diseased Siamese fighting fish showing hemorrhage (A), protruding scales (B) after post-injected with *Aeromonas hydrophila*.

Antimicrobial activity of leave extracts against A. hydrophila

Determination of minimum inhibitory concentration and determination of minimum bactericidal concentration

Five medicinal plants extracts namely *C. asiatica, M. citrifolia, M. officinalis, P. sarmentosum* and *T. catappa* were used in this study. Crude extracts were performed for antimicrobial activities by broth dilution method. The results showed that the Indian almond (*T. catappa*) extract had the best antimicrobial effect which revealed MIC and MBC values as 25 and 12.5 mg/ml, respectively. Consequently, *M. officinalis* and *P. sarmentosum* had moderately antibacterial activity which recorded the MIC and MBC were 12.5 and 25 mg mL^{-1}, respectively (Table 1). Afterwards, *C. asiatica* and *M. citrifolia* showed slightly antimicrobial effect against the *A. hydrophila*. The MIC and MBC of *C. asiatica* and *M. citrifolia* were 50 and 100 mg mL^{-1}, respectively.

Table 1: Antibacterial activity of medicinal plants leave extracts against *Aeromonas hydrophila*

Medicinal plants leave extracts	MIC (mg mL^{1})	MBC (mg mL^{-1})
Centella asiatica	50	100
Morinda citrifolia	50	100
Melissa officinalis	12.5	25
Piper sarmentosum	12.5	25
Terminalia catappa	25	12.5

Discussion

Bacterial diseases are the most important diseases which cause of high morbidity and mortality in ornamental fishes (Barker, 2001). Especially, *A. hydrophila* is ubiquitous bacteria and opportunistic pathogen for immuno compromised fish. (Lewbart, 2008). Moreover, *A. hydrophila* was mainly pathogens has been isolated from diseased ornamental fish (Musa, 2008). According to the results of this study the abnormal symptoms were hemorrhage in the vent, protruding scales, exophthalmia and abdomen distention in experimental fish which corresponded with Lewbart (2008) and Jagoda *et al*., (2014). The

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pathogenicity results showed that *A. hydrophila* was a pathogenic bacteria to the Siamese fighting fish according to the mortality rate of 98.33% when challenged with $7.5x10^7$ cfu $0.05mL^{-1}$. For antimicrobial activity of five medicinal plants against *A. hydrophila* showed that all leaves extracts can be used to treat the *A. hydrophila* infection in the diseased ornamental fish. The MIC of *M. officinalis* and *P. sarmentosum* was 12.5 mg mL^{-1} whereas *T. catappa* at 25 mg mL^{-1} with similar results of Haniffa and Kavitha (2012) which were reported the effective MIC of plant extracts against *A. hydrophila* at MIC value 12.5, 25 and 50 mg mL^{-1}. There have been several reports to determine the antimicrobial effect from the plant sources against the fish pathogens as follow: *Clitoria ternatea* leaves extracts against *A. formicans*, *A. hydrophila*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *P. aeruginosa* and *Streptococcus agalactiae* (Ponnusamy *et al*., 2010). Ruixuan *et al*. (2013) reported that *Psidium guajava* and *Atractylodes lancea* leaves extracts had an antimicrobial effect to *Photobacterium damselae* which isolated from cultured golden pompano (*Trachinotus ovatus*). Vasanthakumar *et al*. (2015) has been revealed the antibacterial activity of *Rosa damascena* petal extracts against *A. hydrophila*. In conclusion, five medicinal plants extracts could be applied in the treatment of bacterial infection caused by *A. hydrophila* for reducing the use of antibiotics in fish populations and the environments.

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