Effect of Cannonball Mangrove *Xylocapus granatum* Koeing Bark Extract on Wound Healing in Siamese Fighting Fish (*Betta splendens*)

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ABSTRACT

Presently, there is significant exploration of natural products as alternatives to chemical drugs, with the goal of mitigating adverse environmental impacts. This study investigates the wound-healing potential of *Xylocarpus granatum* bark extract in Siamese fighting fish, *Betta splendens*, a popular ornamental fish. The extract was prepared using the decoction method. Subsequently, the obtained extract was applied at concentrations of 60, 120, and 180 ppm to treat wounds in betta fish. These treatment groups were compared to a positive control group receiving 15 ppm oxytetracycline and a negative control group receiving no treatment over a 21-day period. The results revealed the fastest healing among groups treated with either 60 or 120 ppm of *X. granatum* bark extract. However, the 60 ppm concentration appeared more promising due to the highest survival rate (85.0±4.3%) among treated fish. Notably, neither the negative control nor positive control groups achieved complete wound healing within 21 days, and these groups had among the lowest survival rates. Regarding the healing effects on various tissue layers, muscle tissue healing was slower in the 60 ppm group compared to the 120 and 180 ppm groups. However, for connective tissue, dermis, epidermis and scale tissues, the 60 ppm treatment group demonstrated the fastest wound healing rates. Based on these findings, *X. granatum* bark extract shows potential as an effective wound healing agent for *B. splendens*, particularly at a concentration of 60 ppm. This natural product could offer benefits for the aquaculture industry and ornamental fish enthusiasts while mitigating environmental impacts.

Keywords: *Betta splendens*, Oxytetracycline, Wound healing, *Xylocarpus granatum*

INTRODUCTION

The utilization of antibiotics and chemical substances in disease treatment may lead to accumulation and persistence of these substances in fish tissues and the surrounding environment, potentially contributing to the emergence of drug resistance (Santoso *et al*., 2013). Vaccination stands as a proven method for disease prevention, albeit with associated costs and effectiveness limited to specific pathogens (Harikrishnan *et al*., 2011). Consequently, there is a growing interest in exploring herbal plants and derivatives as alternative approaches for disease prevention and control in aquatic animals (Reverter *et al*., 2014). One such plant is the Mangrove species *Xylocarpus granatum* Koeing, commonly known as the white mangrove, belonging to the Meliaceae family. This large shrub or tree is typically found in areas with brackish water, such as mangrove forests in the western islands of southern Thailand and the western region of Malaysia. *X. granatum* holds medicinal significance, containing vital substances, including a compound with antifungal properties. This compound has potential applications in wound healing and treating various fungal infections. Notable compounds found in *X. granatum* include alkaloids, condensed tannins, phenolic compounds,

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triterpenes, steroids, cardiac glycosides, and anthraquinones (Polyium, 2018). These compounds have demonstrated efficacy in wound treatment, bacterial elimination (Chitmanat *et al*., 2005), and the eradication of parasites harmful to aquatic animals (Koeypudsa *et al*., 2007).

The Siamese fighting fish or betta is a native fish species in Thailand, holds considerable export value for the country. According to the Department of Fisheries, betta is the top-ranked fish in terms of export value, with an estimated annual export of 1.6 million individuals, valued at 18.4 million baht (Fish inspection Suvarnabhumi, 2021). Key international markets for betta fish include the United States, Japan, Singapore, and France, all of which import bettas from Thailand (Kajitkham, 2000). Betta is not only sought after for export but is also popularly bred for sports and as an ornamental pet, both domestically and internationally (Rodloi, 2011). While historical records indicate that betta fish fighting was once a popular activity, contemporary breeding focuses on achieving aesthetically pleasing appearances for admiration rather than for fighting purposes (Sermwatanakul, 2018). Although the popularity of betta fish fighting has decreased in recent times, there remains a significant number of enthusiasts who continue to show interest in this activity. After betta fish fight, the treatment and wound healing of skin lesions can involve the use of medications or chemicals. In addition to conventional methods, herbal extracts have emerged as an alternative option. Several research studies have shown that herbal plants have immunostimulatory properties, are readily available locally, and are cost-effective (Chitmanat, 2013).

According to the local knowledge of the people in the south of Thailand, *X. granatum* is among the local herb used for wound healing in fishes. However, there is no report on applying this herb to betta, thus this study aims to investigate the healing time and survival rate of betta fish treated with different concentrations of *X. granatum* bark extracts. The utilization of the bark has shown the most promising results in the preliminary tests when compared to the peel and seed, as reported by S. Chingjit (personal communication). The findings from this study hold potential applications in the development of wound healing medications for betta fish.

MATERIALS AND METHODS

Preparation of experimental animals

Male Siamese fighting fish, *Betta splendens*, aged at 8 months, with an average body weight of 2.01±0.96 g and average total length of 5.52±0.39 cm, were housed in glass bottles containing 300 mL of water. Lateral body injuries were induced by gently touching the side of their bodies with a cotton swab soaked in concentrated hydrochloric acid. The fish were left in the glass bottles with a volume of 325 $m \cdot L^{-1}$ to allow the wounds to develop for a period of 72 h. Water exchanges were performed every 24 h, replacing 50 percent of the water, and no food was provided during this process.

Extraction of Xylocarpus granatum bark

The pieces of *X. granatum* bark, measuring approximately 2×4 cm, were dried in a hot air oven at a temperature of 60 ºC for 6 h, then blended into powder. The powder was extracted using the boiling extraction with an ethanol-water mixture (40:60) at a temperature of 60 ºC for 1 h. After cooling down, the extract was filtered through a filter paper no.2 with a pore size of 8 µm, as adapted from Neelapong *et al*. (2019). The final step involved storing the extract in amber glass bottles for experimental use.

Experimental design and data analysis

The experimental design employed in this study was a Completely Randomized Design, consisting of 5 treatments, each conducted with 3 replications, with 10 fish per replicate, resulting in a total of 150 individuals being used. The 5 treatments consisted of: (1) the negative control group without the use of antibiotics; (2) the positive control group, using oxytetracycline at a concentration of 15 ppm; (3), (4), and (5), the treatment groups using *X. granatum* bark extracts at concentrations of 60, 120, and 180 ppm, respectively. Data recording was done daily at 09:00 a.m. to observe wound healing under a light microscope and reported as the number of days until the wound was completely healed. Fish sedation was done using 15 ppm clove oil (Somjai *et al*., 2008). The survival rate over a period of 21 days was also recorded and the survival rate (percentage) was calculated as [number of fish at the end of the experiment/initial number of fish]×100. No water exchanges or feeding were provided during the experiment.

The data were analyzed using Analysis of Variance. The differences between treatments were compared using Duncan's New Multiple Range Test at a significance level of 0.05. The analysis of the data was conducted using SPSS version 23.

RESULTS

The wound healing period and survival rate are presented in Table 1. In the negative and positive control groups (untreated and treated with 15 ppm Oxytetracycline, respectively), the wounds of tested betta fish did not healed within the designated 21 days. Meanwhile, among the groups treated with *Xylocarpus granatum* bark extract, the shortest healing period was observed at a concentration of 60 ppm (11.7±1.7 days), though not statistical different ($p>0.05$) with 120 ppm (12.4±1.1 days). Interestingly, the highest concentration at 180 ppm showed longer ($p>0.05$) period of wound healing (16.2±1.1 days), than the lower concentrations. Regarding the survival rate, betta fish treated with 60 ppm *X. granatum* bark extract exhibited the highest rate at 85.0±4.3%, which was significantly ($p<0.05$) different from both 120 and 180 ppm, where the rates were around 80% with no statistical difference between them. Meanwhile, the negative and positive control groups showed significantly ($p<0.05$) lower survival rate compared to the treatments with extract from *X. granatum* bark, specifically a survival rate of 75.5±6.0% for the group treated with oxytetracycline and 60.1±7.3% for the untreated group.

Table 2 and Figure 1 present the wound healing period in various tissue layers of betta fish over a 21-day treatment period. In the negative control group, only the muscle tissue exhibited recovery. Oxytetracycline remedied all tissues but scale. For the wound healing in each tissue layer, it was found that muscle layer exhibited the shortest recovery period when treated with *X. granatum* bark extract at concentrations of 120 ppm (2.6±0.5 days) and 180 ppm (2.6±0.2 days) ($p>0.05$), which was statistical different ($p<0.05$) from the other treatments. This was significantly ($p<0.05$) different with the groups treated with oxytetracycline (3.8± 0.6 days) and 60 ppm *X. granatum* bark extract (3.4±0.2 days), and the negative control group (9.6±0.1 days).

For the connective tissue layer, treatment with 60 ppm extract resulted in the fastest wound healing period (2.8±0.2 days) and was statistically different from the healing time associated with concentrations of 120 and 180 ppm (3.2±0.2 days and 3.8±0.4 days, respectively). The longest healing time was observed in the oxytetracycline treatment group (4.6±0.2 days).

The results for dermis tissue and epidermis followed a similar trend, wherein the groups treated with 60 ppm and 180 ppm extracts showed shortest healing period (ranging between 1.7±0.5 and 2.4± 0.7 days). Both groups treated with 180 ppm extract and oxytetracycline took a significant longer time for healing, ranging from 2.4±0.2 to 3.5±0.3 days.

Lastly, the scale healing period was shortest in the groups treated with 60 ppm and 120 ppm extracts, taking around 2 days, whereas a significantly longer healing time (4.4±0.5 days) was observed for the group treated with 180 ppm extract.

Table 1. The wound healing period and survival rates of betta fish, treated with various concentrations of *Xylocarpus granatum* bark extracts over a period of 21 days, compared with those of the non-treated group, and the group treated with oxytetracycline.

Parameter	Non-treat	Oxytetracycline 15 ppm	*Xylocarpus granatum* bark extract (ppm)		
			60	120	180
Wound healing (days)	NA	NA	11.7±1.7[a]	12.4±1.1[a]	16.2±1.1[b]
Survival rate (%)	60.1±7.3[d]	75.5±6.0[c]	85.0±4.3[a]	80.3±5.7[b]	80.1±2.8[b]

Note: Mean±SD in each row superscripted with different lowercase letters are significantly ($p<0.05$) different; NA = Wound unhealed

Table 2. The wound healing period observed in various tissue layers of betta fish, treated with various concentrations of *Xylocarpus granatum* bark extracts over a period of 21 days, compared with those of the non-treated group, and the group treated with oxytetracycline.

Wound healing (days)	Non-treat	Oxytetracycline 15 ppm	*Xylocarpus granatum* bark extract		
			60 ppm	120 ppm	180 ppm
Muscle	9.6±0.1[c]	3.8±0.6[b]	3.4±0.2[b]	2.6±0.5[a]	2.6±0.2[a]
Connective tissue	NA	4.6±0.2[c]	2.8±0.2[a]	3.2±0.2[b]	3.8±0.4[b]
Dermis	NA	3.5±0.3[b]	1.7±0.5[a]	2.4±0.7[a]	3.1±0.6[b]
Epidermis	NA	2.5±0.5[b]	1.7±0.6[a]	2.0±0.3[a]	2.4±0.2[b]
Scale	NA	NA	2.3±0.3[a]	2.2±0.2[a]	4.4±0.5[b]

Note: Mean±SD in each row superscripted with different lowercase letters are significantly ($p<0.05$) different; NA = Wound unhealed

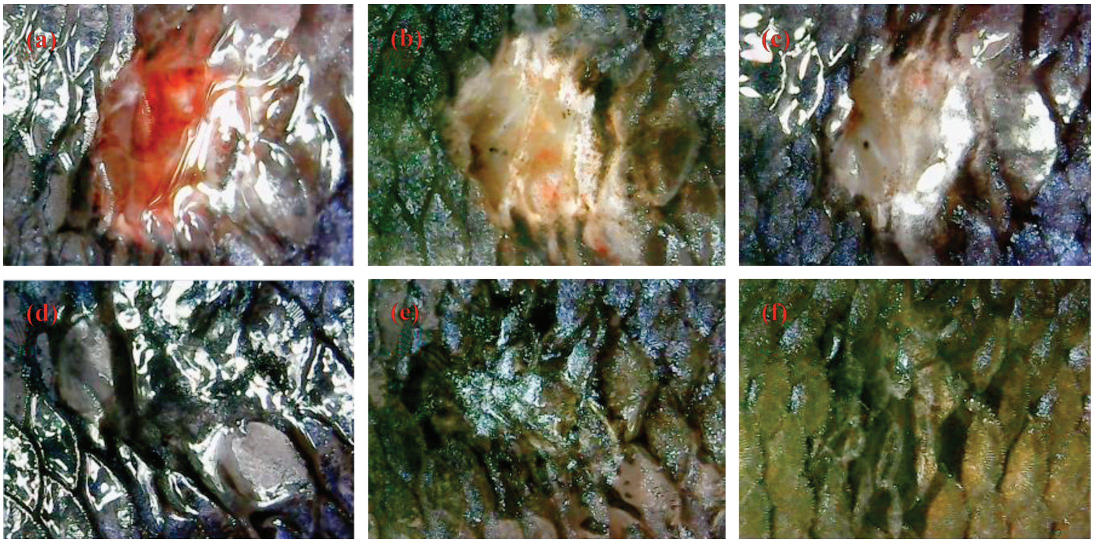

Figure 1. The images depict wound healing outcomes in various tissue layers of betta fish treated with *Xylocarpus granatum* bark extract; a) Healing of a new wound in muscle layer; b) Connective tissue healing; c) Dermis healing; d) Epidermis healing; e) Scale healing; and f) Completely healing.

DISCUSSION

The utilization of *Xylocarpus granatum* as a medicinal plant among coastal communities suggests its promising role as a potential pharmaceutical resource (Saptiani *et al*., 2019). Traditional medicinal applications encompass a wide range of conditions, including fever, malaria, inflammatory issues, dysentery, diarrhea, cholera, gastrointestinal ailments, diabetes, elephantiasis, as well as qualities related to antimicrobial, antioxidant, anticancer, antidislipidemia, and cardiotonic effects (Yoswaty *et al*., 2021).

The effective wound treatment in betta fish treated with extract from *X. granatum* bark is likely due to the presence of tannins in the extract, known for their antiseptic properties and ability to prevent bacterial decay (Pimolrat *et al*., 2017). Tannins also possess antibacterial properties (Chung and Chou, 1998). Tannic acid has the ability to impede the proliferation of intestinal bacteria through its strong affinity for metal ions, especially iron, leading to the formation of a chelate. This chelate, akin to a siderophore, exerts toxicity upon microorganism membranes. When tannins create a chelating complex with iron within the environment, this process deprives microorganisms of essential iron required for growth under aerobic conditions. The bacterial growth is hindered, partly attributed to disruptions in ribonucleotide precursor reduction for DNA, the synthesis of heme, and other vital cellular mechanisms (Scalbert, 1991). Tannins have the ability to improve water quality and reduce bacterial contamination within 21 days, without affecting temperature, pH value, oxygen solubility, phosphate solubility and reducing ammonia and nitrite levels (Puttamat and Worawat, 2007).

Similar results were observed with the use of extract from Iron Wood (*Hopea odorata* Roxb.) seeds at a concentration of 5 ppm and oxytetracycline at a concentration of 5 ppm, which healed wounds within 13 and 12 days, respectively (Chanmanee, 2020). Furthermore, the use of extract from *Aloe vera* (L.) Burm.f. at concentrations of 100–200 ppm resulted in the normal healing of wounds in hybrid catfish within only 6 days, compared to 15 days without treatment. The extract was also found to be an effective alternative to oxytetracycline (Boonma *et al*., 2021). Similarly, it was found that the use of extract from neem (*Azadirachta indica*) seed oil, kalojira (*Nigella sativa*) seed oil, neem leaf extract and mahogoni (*Swietenia mahagoni*) seed oil can treat fish disease (Alam *et al*., 2014) and reduce the size of skin lesions (Julinta *et al*., 2019).

CONCLUSION

Xylocarpus granatum bark extract, obtained through the decoction method, demonstrates effective potential in treating injuries in betta fish at a concentration of 60 ppm. To enhance its viability for commercial use, it is recommended to refine the extraction process ensuring the production of a pure extract. This refinement is crucial for accurately determining the exact concentration needed for further development and commercial applications.

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