In vitro screening of *Sonneratia alba* extract against the oomycete fish pathogen, *Aphanomyces invadans*

Afzali S.F.; Wong W.L.*

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Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, Kampar 31900, Perak, Malaysia.
*Corresponding author's Email: wongwl@utar.edu.my

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Introduction

Epizootic ulcerative syndrome (EUS), caused by the aquatic oomycete fish pathogen, *Aphanomyces invadans* (David and Kirk, 1997) is one of the OIE-listed diseases that leads to huge economic losses in the fish industry in the world. Traditional disease management strategies relying on chemotherapy continue to cause undesirable effects such as antibiotic resistance, environmental pollution and food security issues (Pandey *et al.*, 2012). In addition, there has been no vaccine available to prevent the outbreak of EUS according to the Office International des Epizooties (OIE) (2015). Therefore, many current researches focus on alternative strategies such as using plants for EUS treatments (Fairweather, 1999; Campbell *et al.*, 2001; Chowdhury and Rahman, 2013; Alam *et al.*, 2014; Uthayakumar *et al.*, 2014; Kumar *et al.*, 2015; Yogeshwari *et al.*, 2015).

Generally, biological compounds are known to be easily biodegradable, environmentally friendly, inexpensive, renewable and locally available, and can be easily prepared (Ortuño *et al.*, 2002; Valladão *et al.*, 2015). More than 400,000 species of tropical plants are known to have medicinal properties (Citarasu, 2010). Biomedicinal plants such as mangrove plant extracts or their by-products contain several active compounds such as phenols, polyphenols, alkaloids, quinones, terpenoids, lectines, and polypeptides that have been shown to be effective

alternatives to traditional chemotherapies and vaccines for disease treatments and control (Citarasu *et al.,* 2002; Sivaram *et al.,* 2004; Kjer *et al.,* 2009; Citarasu, 2010; Harikrishnan *et al.,* 2011). A number of plants, such as *Mikania cordata* (Burm), *Rauvolfia tetraphylla* L., neem (*Azadirachta indica* A. Juss.), akand (*Calotropis gigantea* L.), turmeric (*Curcuma longa* L.), *Curcuma zedoaria* (Christm.), Indian sorrel (*Oxalis corniculata* L.) and *Nigella sativa* L.' have been proved to enhance disease resistance of fish against EUS (Campbell *et al.,* 2001; Chowdhury and Rahman, 2013; Kumar *et al.,* 2015; Yogeshwari, *et al.,* 2015). However, most of these plants are required in relatively high amounts to show effectiveness in the treatment of EUS infected fish.

The present study is the first attempt in applying the mangrove plant, *Sonneratia alba* as a biomedicine against EUS causative Oomycete fungus. *Sonneratia alba* (Smith) is a mangrove plant belonging to the family Sonneratiaceae. It is known as mangrove apple or "perepat" in Malaysia (Duke and Jackes, 1987). Members of this family are rich in cyclitol, polyol, sucrose, glucose, fructose, condensed and hydrolysable tannins, minerals, nucleotides (Bandaranayake, 2002). Tannin is known for its antimicrobial activity and probably evolved in plants as a defense against microbial attack (Balasooriya *et al.,* 1982). In the present study, we aimed to investigate the *in vitro* antifungal effects of the methanol extract of *S. alba* against *A. invadans* using the disc diffusion methods.

Materials and methods

The leaves of *S. alba* were collected from Sungai Prai in Penang, Malaysia and identified according to Duke and Jackes (1987) (Fig. 1). The fresh leaves were washed with distilled water, blotted dried and freeze-dried using a freeze dryer (Martin Christ, Germany). The dried leaves were then powdered using a mechanical grinder and weighed. The methanol extraction of dried leaves was performed using a Soxhlet extraction device. The extract was distillated using a rotary evaporator (Büchi, Switzerland) and stored in air tight containers at -20°C until required. The agar disk diffusion technique was conducted according to Bailey (1983). Briefly, the *A. invadans* strain (NJM9701) (courtesy of Dr. Oidtmann B, U.K.) was inoculated onto a sterilized glucose peptone (GP) agar media (3 gL^{-1} glucose , 1 gL^{-1} peptone, 0.128 gL^{-1} $MgSO_4.7H2O$, 0.014 gL^{-1} KH_2PO_4, 0.029 gL^{-1} $CaCl_2.2H_2O$, 2.4 mgL^{-1} $FeCl_3.6H_2O$, 1.8 mgL^{-1} $MnCl_2.4H_2O$, 3.9 mgL^{-1} $CuSO_4.5H_2O$, 0.4 mgL^{-1} $ZnSO_4.7H_2O$, 12 gL^{-1} technical agar, 10 mL L^{-1} Penicillin) plate and incubated for five days at 25°C for preparation of agar discs. A set of three dilutions (100, 500 and 1000 ppm) of *S. alba* extract was prepared with distilled water.

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Figure 1: ***Sonneratia alba*** **mangrove plant tree can be morphologically characterized by rounded leaf tips (arrow), corolla width flattened fruits (circles), and flower with white and small petals (rectangle).**

Distilled water was used as a positive control. The agar diffusion discs experiments were prepared by soaking the pre-inoculated agar discs (6 mm in diameter) individually in different concentrations of 100 µl of *S. alba* extract. The discs were soaked in the extracts for one hour at 25°C. The treated discs were then washed three times in the distilled water and placed on the GP agar plates and incubated for seven days at 25ºC. Mycelial growth of *A. invadans* was monitored daily for seven days. The antifungal activities of the extracts were evaluated by measuring the mycelial growth (mm) from the edge of the pre-inoculated discs on the GP plates. The boundary dilution without any visible growth was defined as the minimal inhibitory concentration (MIC) for *A. invadans* at the given *S. alba* extract concentrations. The filter paper disk diffusion method (Kohner *et al.*, 1994) was applied to compare the inhibition zone of *S. alba* extracts with commercially available antifungal agents, namely malachite green, known to be the most effective drug against EUS, and formalin at their minimal inhibiting concentration for *A. invadans* mycelia growth. Sterile filter papers (Whatman, No. 4) with a diameter of 5 mm were impregnated with 100 µL of each of the three dilutions of extract solution and placed on the GP agar plate including a pre-inoculated agar cube (10 mm) in the center which was incubated for seven days at 25ºC to monitor the mycelia growth of *A. invadans.* All the experiments were performed in triplicate.

Results and discussion

The methanol extract of *S. alba* effectively inhibited the mycelial growth of *A. invadans* at a minimum concentration of 1000 ppm for both the agar and filter paper diffusion experiments. In the agar diffusion test,

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500 ppm of the extract inhibited the fungus mycelial growth up to 96 hours. The mycelial growth from the edge of the pre-inoculated *A. invadans* agar discs treated with *S. alba* extracts at concentrations of 100, 500 and 1000 ppm were 15, 8 and 0 mm respectively (Fig. 2). The results of the filter paper disc test showed that the *S. alba* extract at its minimal inhibitory concentration (1000 ppm) has similar qualitative inhibitory effects as malachite green at 1 ppm and formalin at 250 ppm (Fig. 3).

The antimicrobial activity of *S. alba* extract was previously reported by Saad *et al.* (2012) who suggested that this plant extract may possess therapeutic property against human pathogens and infectious diseases caused by *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. However, the authors had noted that *S. alba* extract was not sensitive even at high concentration (10,000 ppm) against the tested fungal strain, *Candida albicans*, and yeast, *Cryptococcus neoformans*.

Campbell *et al.* (2001) had tested several plant extracts and showed that high concentrations (5000-10000 ppm) of herbals such as turmeric (*Curcuma longa*), propolis resin, *Calophyllum inophyllum* L., *Eugenia caryophyllus* L., and *Ficus pumila* L. are effective in inhibiting the mycelial growth of *A. invadans in vitro*. However, the application of high concentration of herbals seems not to be practical due to the expenses may be imposed.

It is reported that neem, *A. indica*, extract (azadirachtin) may inhibit *A. invadans* sporulation at 1000 ppm *in vitro* (Campbell *et al.*, 2001), and possesses antifungal effect on EUS-affected fish (Harikrishnan *et al.*, 2005; Chowdhury and Rahman, 2013) as well.

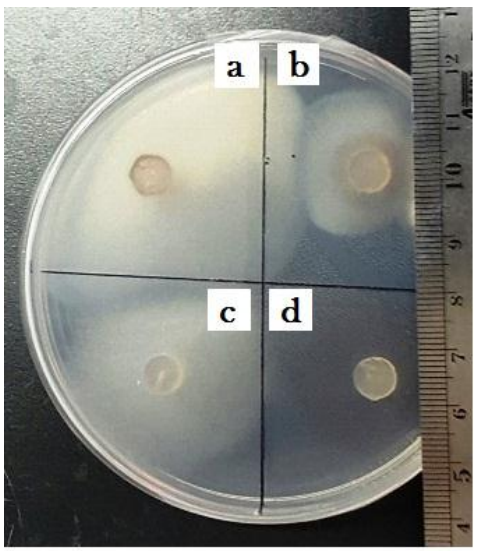

Figure 2: Mycelial growth of pre-inoculated *Aphanomyces invadans* agar discs treated with different concentrations of *Sonneratia alba* extract and distilled water on a glucose peptone plate. a) Distilled water (positive control); b) *Sonneratia alba* extract of 500 ppm; c) *Sonneratia alba* extract of 100 ppm; d) *Sonneratia alba* extract of 1000 ppm.

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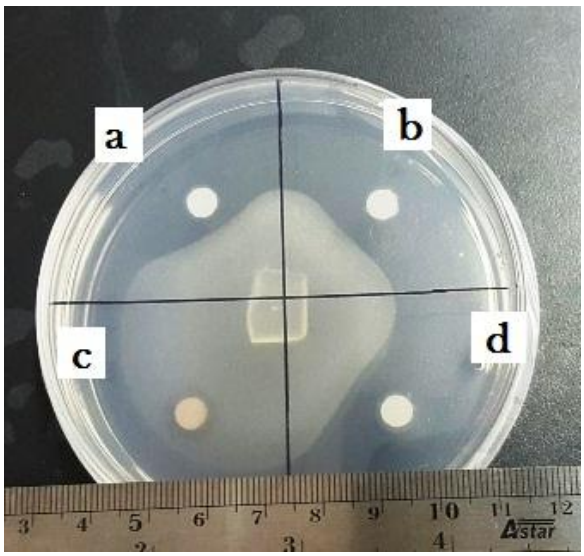

Figure 3: Inhibition zone of mycelial growth of *Aphanomyces invadans* resulting from malachite green, formalin and different concentrations of *Sonneratia alba* extract on the filter paper discs. a) formalin at 250 ppm; b*) Sonneratia alba* extract at 1000 ppm; c) *Sonneratia alba* extract at 500 ppm; d) Malachite green at 1 ppm.

Campbell and his colleagues (2001), also tested other herbal extracts including *Psidium guajava*, *Phyllanthus urinaria*, *Phytolacca americana*, garlic clove, *Acalypha lanceolata*, *Acalypha indica*, tea tree oil and witch-hazel that did not show any inhibitory effects against *A. invadans* even at higher concentration (5000 – 1000000 ppm), and cannot be recommended as prophylactic measure against EUS. In another study, Fairweather (1999) found that administration of D-limonene, a natural compound found in orange peels with anti-cancer properties (Sun, 2007), is totally ineffective on *A. invadans* growth at all concentrations tested *in vitro*. Even though malachite green and formalin were reported to be able to inhibit the mycelial growth of *A. invadans* at 1 ppm and 250 ppm, respectively (Campbell *et al.*, 2001), these chemicals are toxic and malachite green, for example, is forbidden by U.S. food and drug administration (FDA) in aquaculture (Khan *et al.*, 2011). The present study suggests that *S. alba* methanol extract with a concentration of 1000 ppm may be used as an alternative in controlling the invasive growth of the EUS causative agent, *A. invadans*. This can be exploited further in the development of potent antifungal compounds.

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