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Original Article

Betel leaf extract as an antifungal agent for swamp fish eggs: A case study of *Saprolegnia* sp on kissing gourami (*Helostoma temminckii*) and climbing perch (*Anabas testudineus*) fish

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Abstract

Several indigenous swamp fish species have been successfully domesticated and cultivated by fish farmers in Sumatera, Indonesia. However, the hatching phase in hatcheries commonly encounters fungal infections attributed to *Saprolegnia* sp. This study investigates the efficacy of betel leaf extract in protecting the eggs of kissing gourami and climbing perch from saprolegniasis. Mimicking natural infection conditions, all eggs were exposed to 5 artificially infected eggs per tank. Prior to infection, the water was treated with betel leaf extract at various concentrations. Results indicate that betel leaf extract significantly protects the eggs from this fungal infection. For the climbing perch, the optimal dosage was determined to be 300 mg L^{-1}, resulting in a hatching rate of 90% and a prevalence rate of 10%. For the kissing gourami, the optimal dosage was found to be 120 mg L^{-1}, resulting in a hatching rate of 86.66% and a prevalence rate of 13.34%.

Keywords: betel leaf extract, antifungal, fish eggs, climbing perch, kissing gourami, saprolegniasis

1. Introduction

Saprolegniasis, caused by *Saprolegnia* sp., commonly referred to as water molds and classified within the oomycetes group, manifests as a fungal disease primarily affecting fish eggs, posing a significant threat to hatchery operations and resulting in potential economic losses (Kumar, Mandal, Bulone, & Srivastava, 2020; Pavić *et al.*, 2022; van den Berg, McLaggan, Diéguez-Uribeondo, & van West, 2013). Traditionally, fish farmers have resorted to employing the chemical malachite green to combat saprolegniasis outbreaks. However, the utilization of malachite green is associated with adverse environmental effects and human health risks due to its mutagenic and carcinogenic properties (Srivastava, Sinha, & Roy 2004). Consequently, there arises a pressing need for alternative anti-fungal agents that are more environmentally sustainable, such as plant extracts.

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Betel leaf, *Piper betle* Linn, is a medicinal plant tissue that has several effects such as antifungal, antibacterial, anti inflammatory, antioxidant, and ability to eliminate free radicals (Madhumita, Guha, & Nag, 2020; Nayaka *et al.*, 2021; Nur *et al.*, 2017). As an antifungal agent, betel leaf contains tannins, phenols, and other biological compounds that very strongly act against some fungi. For example, the inhibitory zone of betel leaf was twofold that of fluconazole against *Candida albicans (*Sivareddy *et al.*, 2019*).* However, some factors affect the compound concentration profile in betel leaf, such as plant variety, climate, and geographical location. Besides, utilization of betel leaf extract as an antifungal agent in each fish species might need different practices, due to fish size, development stage, and physiological properties, among others. Thus, experimental exploration is needed to get the optimum dosage of betel leaf extract for each fish species. Some studies on betel leaf extract have been done for silver barb fish eggs, *Barbonymus gonionotus*, giant gourami eggs, *Osphronemus gouramy*, zebra fish eggs, *Danio rario*, and milk fish eggs, *Chanos (*De Vera, De Castro, & Dulay, 2016; Ghofur, Sugihartono, & Thomas, 2014; Susanti & Nugroho, 2022; Susilo & Yusanti, 2022*).* Based on those, the dosage and the extraction method will have different effects in different fish species.

The experiments conducted in this study pertained to applied research aimed at benefitting swamp fish farmers, particularly in the region of Sumatera, Indonesia, which possesses abundant natural resources in the form of swampy terrain suitable for development. It is imperative to ascertain the appropriate dosage of betel leaf extract for economically viable swamp fish cultivation, notably of species like climbing perch and kissing gourami, in order to mitigate potential losses in hatchery production. The primary objective of this research is to elucidate the efficacy of betel leaf extract in controlling saprolegniasis in fish eggs, thereby contributing valuable insights to the field.

2. Materials and Methods

The experiment comprised two distinct phases. Initially, the toxicity of betel leaf extract was assessed across a range of concentrations spanning from 100 to 1000 mg L^{-1}. Subsequently, utilizing the 50% lethal concentration for a duration of 24 hours (LC_{50} 24h) as a reference point, three concentrations were selected to identify the optimal dosage for the treatment of climbing perch and kissing gourami eggs.

2.1 Betel leaf extraction

Betel leaves were sourced from a local market in South Sumatera, Indonesia, and underwent a meticulous washing process before being dried at 40°C for 24 hours. This drying procedure adhered to the protocol outlined by Anggraini & Masfufatun (2017). Following drying, the leaves were finely pulverized, and 200 g of the resulting powder was subjected to maceration extraction in 1 L of 96% ethanol for 24 hours. The resulting extract was subsequently filtered through Whatman filter paper No. 42 to eliminate any solid particulates. Further concentration of the extract was achieved using a rotary evaporator operating at 50°C until the extract reached a jelly-like consistency.

2.2 Spawning fish brood-stocks and preparing fish eggs

Semi-artificial spawning was conducted for kissing gourami and climbing perch fish using an intramuscular injection of 0.5 ml Kg^{-1} of Ovaprim, a commercially available solution containing OvaRH and a dopamine inhibitor. The sex ratio was maintained at 2:1 (male:female), with climbing perch weighing 20-30 g/fish and kissing gourami weighing 50-80 g/fish. The broodstocks were individually reared in glass tanks measuring 25x25x25 cm^3 and provided with aeration. Following fertilization, all eggs were promptly collected and transferred into small conical tanks for further development.

2.3 Toxicity assay

Fish eggs (n=50 per tank) were subjected to exposure to varying concentrations ranging from 100 to 2000 mgL^{-1} of betel leaf extract for a duration of 24 hours to ascertain the LC_{50} (concentration at which 50% of the population succumbs). The LC_{50} was calculated according to the formula outlined by Reed & Muench (1983):

$$\text{Proportionate distance (PD)} = \frac{\text{\%infected at dilutin next above 50\% - 50\%}}{\text{(\%infected at dilutin next above 50\% - (\%infected at dilutin next below 50\%)}}$$

The LC_{50} was then calculated based on the following formula:

log 50% end point=(log dilution above 50%) – (PD × log dilution factor)

Dilution factor is the fold difference between the concentration for above 50% and below 50% infected.

2.4 Determination of optimal dosage for fish eggs

Following the toxicity test, the highest dosage that did not induce mortality in the eggs was identified as the midpoint of treatment. Subsequently, one dosage lower and one dosage higher than the midpoint were selected for further investigation. For climbing perch, the midpoint treatment was determined to be 400 mg L^{-1}, leading to the selection of optimal dosage candidates at 300 mg L^{-1}, 400 mg L-1, and 500 mg L^{-1}. Similarly, for kissing gourami, the midpoint treatment was established as 160 mg L^{-1}, resulting in optimal dosage tests for 120 mg L^{-1}, 160 mg L^{-1}, and 200 mg L^{-1}. All the experiments included also positive and negative control groups, each replicated three times.

2.5 Preparation of fungal infection

The artificial initiation of an infection was achieved by submerging unfertilized eggs in water containing *Saprolegnia* sp., sourced from the pathogen collection at the Laboratory of Aquaculture, Sriwijaya University. *Saprolegnia* sp. was cultured on Potato Dextrose Agar (PDA, Sigma Aldrich) for 24 hours at room temperature prior to inoculation. Following cultivation, agar segments measuring 2x2 cm^2, harboring *Saprolegnia* hyphae, were excised and introduced into the tank containing unfertilized eggs, resulting in the

คแcomprehensive infection of all eggs by *Saprolegnia* sp. Prior to the commencement of treatment, the tank was initially seeded with 5 infected eggs, followed by the immersion of an additional 50 eggs in specified concentrations, with subsequent rearing conducted until hatching ensued.

2.6 Hatching rate, hatching period and prevalence of fungal infection

The hatching rate was observed after eggs hatched and calculated by use of the following formula:

$$\text{Hatching rate (\%)} = \frac{\text{Number of hatching eggs}}{\text{Total number of eggs}} \text{ X } 100\%$$

The calculation of hatching period was taken from 60% of total eggs that hatched from each treatment. The length of time from eggs (HT) is known by calculating the time when fertilization occurs (HT_0) until the eggs hatched for 60% (HT_n) with the following formula:

$$HT = HT_n - HT_0$$

The saprolegniasis level or the prevalence of infection in eggs were calculated by counting the number of dead eggs, which were overgrown by fungi; and dead eggs which were not overgrown by fungi, as follows:

$$\text{Prevalence (\%)} = \frac{\text{Dead eggs over grown by fungi}}{\text{Total number of eggs}} \text{ X } 100\%$$

2.7 Water quality

During treatment, water quality was monitored and maintained in optimum range for egg incubation as regards temperature, pH, and dissolved oxygen.

2.8 Data analysis

The experimental data are represented in terms of means and standard deviations. Subsequently, one-way analysis of variance (ANOVA) was conducted to analyze variations and for comparison of treatment groups with different betel leaf concentrations, employing a significance level of $P< 0.05$.

3. Results and Discussion

3.1 Toxicity assay

Betel leaf extracts are well-known for their potential biological activities against various pathogens. However, due to the complex composition of herbal extracts, including betel leaf extract, it remains crucial to assess their toxicity prior to application in aquaculture. In a herbal toxicity evaluation, the determination of the median lethal concentration or LC_{50} is imperative. LC_{50} represents the concentration of a medicinal agent that, when administered as a single exposure over a predetermined brief period, results in the mortality of 50% of the test animals, such as fish or fish eggs. It is evident that determining the LC_{50} serves as an essential tool to establish safe concentrations based on ecotoxicological considerations before the utilization of herbal extracts in aquaculture practices (Semwal, Kumar, Kumar, & Singh, 2023; Yunus, Mardhiah, & Jhon, 2019).

A significant relationship was observed between the concentration of betel leaf extract and the mortality of fish eggs. Morphologically, kissing gourami eggs are larger than climbing perch eggs, suggesting that the lethal concentration of betel leaf extract differs between the two species. The diameter of the kissing gourami**'**s eggs ranged from 1.0 to 1.2 mm while for climbing perch it was around 0.6 to 0.8 mm. The specific size for both fishes can vary depending on factors such as the individual specimens and environmental conditions (Helmizuryani, Djumanto, Muslimin, Aminah, & Khotimah, 2020; Helmizuryani, Suwigyo, Hanafiah, & Faizal, 2021; Rahmadi, Syahril, Nur, Maulida, & Muchlisin, 2021). The LC_{50} values for kissing gourami eggs and climbing perch eggs were determined to be 510.505 mg L^{-1} and 1819.35 mg L^{-1}, respectively (Table 1 and Table 2).

3.2 Hatching rate, hatching period, and prevalence of fungal infection

The hatching rate demonstrated a general improvement compared to the positive control group in both

Table 1. Calculation of lethal concentration (LC_{50}) for climbing perch eggs immersed in betel leaf extract for 24 hours

No	Dose (mg L^{-1})	Log dose (mg L^{-1})	Number of mortality	Number of survival	Mortality (%)
1	100	2.00	0	50	0
2	200	2.30	0	50	0
3	300	2.48	0	50	0
4	400	2.60	0	50	0
5	500	2.70	7	43	14
6	1000	3.00	17	33	34
7	1500	3.18	20	30	40
8	2500	3.40	34	16	68

Calculation of lethal concentration (LC_{50}) using Reed-Muench (1983) Method:
(50 – 40) ÷ (68 – 40) = 0.36
3.40 - 3.18 = 0.22
0.36 x 0.22 = 0.0792
3.18 + 0.0792 = 3.2592
Antilog of 3.2592= 1819.35

Table 2. Calculation of lethal concentration (LC_{50}) for kissing gourami eggs immersed in betel leaf extract for 24 hours

No	Dose (mg L^{-1})	Log dose (mg L^{-1})	Number of mortality	Number of survival	Mortality (%)
1	80	1.90	0	50	0
2	160	2.20	0	50	0
3	240	2.38	8	42	16
4	320	2.51	12	38	24
5	400	2.60	16	34	32
6	520	2.72	26	14	52
7	600	2.78	30	20	60
8	800	2.90	50	0	100

Calculation of lethal concentration (LC_{50}) using Reed-Muench (1983) Method:
(50 – 32) ÷ (52 – 32) = 0.9
2.72 – 2.60 = 0.12
0.9 x 0.12 = 0.108
2.60 + 0.108 = 2.708
Antilog of 2.708= 510.505

fish eggs (Figure 1). Concurrently, the prevalence rate suggests a correlation between fungal infection and reduced hatching rates of the eggs and longer hatching period. Consequently, the positive control group, characterized by a higher incidence of *Saprolegnia* sp. infection, exhibited lower hatching rates and longer hatching time. The results indicate that the concentration of betel leaf extract impacts the hatching rate, hatching period and prevalence rate of both kissing gourami and climbing perch eggs.

The findings of this study suggest that while the lowest concentration within each group tends to produce effects similar to those of the control group, higher concentrations of the extract do not necessarily accelerate the hatching period (Table 3). Based on Hassan *et al.* (2018) hatching period for climbing perch is 18 hours post fertilization (hpf) under temperature 28.5 ± 0.4°C, pH 7.13 ± 0.90, and dissolved oxygen 5.06 ± 0.38 mg l^{-1}. Similar result was also reported by Loh & Ting (2015) whomentioned the fish larvae hatched at 17 – 18 hpf under conditions of a recirculating system with temperature at 27.3 ± 1 °C, pH at 7.5 ± 0.5, and DO at 6.8 ± 1.5 mg L^{-1}. For kissing gourami, Wahyuningtias *et al.*, (2015), reported the hatching period was around 20 hpf. In this current experiment, the hatching period of fish eggs was around 20 hpf, which was longer than the normal hatching time. Thus, the fungal infection was able to alter the metabolism of fish eggs and affect the hatching period. Indeed, environmental factors predominantly exert a significant influence on hatching, notably the timing thereof. Temperature, recognized as a primary factor, has the capacity to either accelerate or delay the hatching. Additionally, other parameters such as water pH, dissolved oxygen level, chemical composition, light exposure, salinity, and toxins may contribute to delayed hatching, as these factors have the potential to alter the metabolic processes of eggs (Korwin-Kossakowski, 2012). Moreover, it is important to consider the characteristics of the eggs, including their size and chorion composition, as these factors can significantly impact early development. Each species may exhibit distinct characteristics in their chorion composition, which can in turn affect hatching time and hatchability. While there is limited information available on the egg characteristics of these fish species, our results indicate a correlation between betel leaf extract concentration and hatching period, highlighting the potential influence of the extract on embryonic development in these fish species (Blaxter, 1988; Pérez-Atehortúa *et al.*, 2023).

While the exact pathogenesis of this fungal infection remains uncertain, it is hypothesized that hyphae may compromise egg viability by encroaching upon and enveloping the embryo membrane, thus disrupting its osmoregulatory functions (Liu *et al.*, 2014). The determination of the optimal concentration of betel leaf extract gave 120 mg L^{-1} for kissing gourami and 300 mg L^{-1} for climbing perch, underscoring the critical role of *in vivo* assessment in evaluating antifungal efficacy. While *in vitro* assays often suggest a direct relationship between higher concentrations of antifungal agents and enhanced protective effects, the translation to *in vivo* settings can be nuanced. This disparity is exemplified by observations where, despite higher concentrations exhibiting superior protection in vitro, the biological constraints inherent to the eggs can impede hatching rates. For instance, in the case of *Kaempferia galanga* extract, optimal protection of catfish eggs against saprolegniasis was achieved at 60 mg L^{-1}, whereas concentrations exceeding 80 mg L^{-1} resulted in a notable decline in hatching rates (Humsari, Rosidah, & Junianto, 2017).

Similar endeavors to combat saprolegniasis in fish eggs using plant extracts have been documented across various species, encompassing both in vitro and in vivo investigations, such as *Terminalia catappa* extract for angelfish and *Opuntia stricta* extract for pikeperch, among others (Ben Khemis, Besbes, Hamza, M'Hetli, & Sadok, 2016; Meneses *et al.*, 2022; Mostafa, Al-Askar, & Taha, 2020; Sudarno, Hakim, & Kusdarwati, 2017). Thus, the determination of the optimal concentration of plant extract remains paramount for each species, as it must account for diverse factors that influence the efficacy of antifungal agents in aquaculture practices.

3.4 Water quality

During incubation, water quality parameters were monitored to maintain optimal conditions for fish eggs. Generally, swamp fish inhabit environments characterized by temperatures ranging from 28 to 30°C, pH levels between 5 and 6.5, and dissolved oxygen concentrations of 3.5 to 5 mg

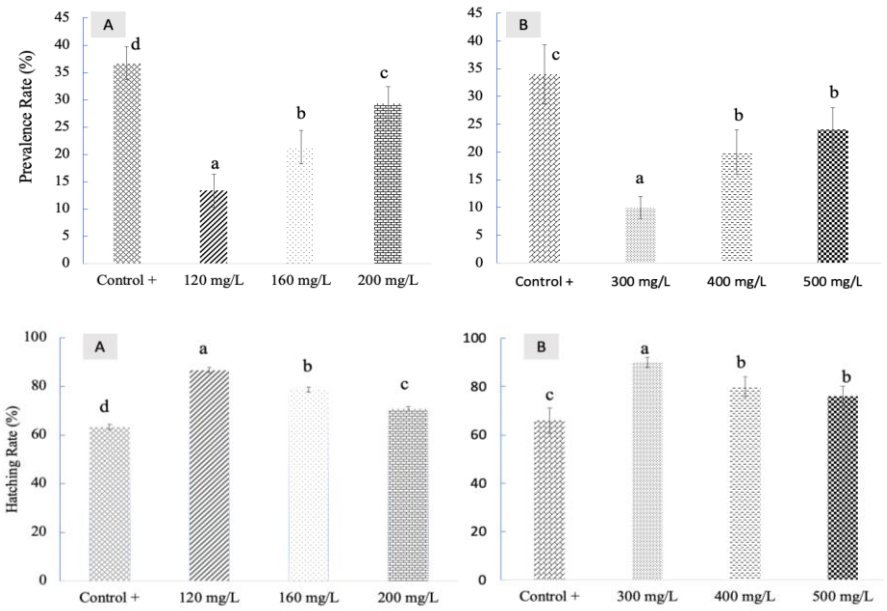

Figure 1. Hatching rate and Prevalence rate. (A) Kissing gourami; and (B) climbing perch. Values with the same letter have no statistically significant difference, based on the Least Significance Difference test at α = 5%.

Table 3. Hatching period of Climbing perch and Kissing gourami eggs

Climbing perch		Kissing gourami	
Group	Duration (minutes)	Group	Duration (minutes)
Control	1390 ±30.00^{d}	Control	1394±17.6^{d}
300 mg L^{-1}	1320 ±15.27^{c}	120 mg L^{-1}	1317±12.5^{c}
400 mg L^{-1}	1200 ±37.75^{a}	160 mg L^{-1}	1255±24^{b}
500 mg L^{-1}	1260 ±27.84^{a}	200 mg L^{-1}	1170±22.3^{a}

Note: Values followed by the same letter have no statistically significant difference, based on Least Significance Difference test at α=5%.

L^{-1}. Studies by Rahmadi *et al.* (2021) and Zulfikar, Muhammad, & Slamat, (2020), have indicated that for climbing perch embryogenesis, the preferred temperature falls within the range from 25°C to 30°C, with pH levels maintained between 6.5 and 7.5, and dissolved oxygen levels above 5 mg L^{-1}. Despite instances of dissolved oxygen levels during experimentation being less than 5 mg L^{-1}, all uninfected eggs successfully hatched. Optimal conditions for kissing gourami, as outlined by Helmizuryani *et al.* (2021), involve pH levels ranging from 3 to 4.5, dissolved oxygen concentrations from 3 to 5.6 mg L^{-1}, and temperatures ranging from 24°C to 28°C. Fluctuations in environmental conditions can potentially impact egg development and necessitate energy allocation for homeostatic processes. Consequently, regular monitoring of water parameters such as temperature, pH, and dissolved oxygen levels throughout experimentation remains imperative to ensure the optimal development of eggs.

4. Conclusions

Piper betel leaf extract was able to protect climbing perch eggs and kissing gourami eggs against *Saprolegnia* sp infection. This research revealed that by species, the optimal concentration of betel leaf extract as an antifungal agent can differ, as it was 120 mg L^{-1} and 300 mg L^{-1} for kissing gourami and climbing perch eggs, respectively.

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