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Research Article
Effects of Extract of Betel Leaf (Piperaceae) against Ectoparasite "Ich" on Pangasius Catfish

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Abstract
Background and Objective: Freshwater fish aquaculture in Indonesia has grown rapidly, especially the aquaculture of catfish (*Pangasianodon hypophthalmus*). This species is very good because it is fast-growing and very popular in the market and is important for national food security in many Asian countries. One of the problems faced by freshwater fish aquaculture is ectoparasite *Ichthyophthirius multifiliis* infection, which often results in significant economic losses to freshwater fish aquaculture. This study aimed to check the effect extract of betel leaf against the ectoparasite, *Ichthyophthirius multifiliis* in pangasius catfish in an eco-friendly manner. **Materials and Methods:** A total of 120 fishes with a mean weight of 4.17 ± 0.96 g and a length of 8.5 ± 0.67 cm were examined. Preliminary research was carried out to detect ectoparasites in fish. All fish was infected with ectoparasitic Ich (100%) and were identified as a salt-like granule white spot and a large C-shaped macronucleus. Infected fishes were transferred and equally distributed to the tank (20 L water) which had previously been treated with betel leaf extract for 24 hrs, 3 days, at doses 2.5, 5 and 7.5 g L^{-1} and control. **Results:** The results showed that the betel leaf extract solution effect decreased significantly to the number of ectoparasites *Ichthyophthirius multifiliis*, both in mucus and pangasius catfish and a dose of 7.5 g L^{-1} was the optimum dose. **Conclusion:** Betel leaf extract has the potential to control the decrease in the number of ectoparasites, though further phytochemical studies will need to be performed.

Key words: Aquaculture, betel leaf extract, ectoparasite, granule white spot, *Ichthyophthirius multifiliis*, *Pangasianodon hypophthalmus*, Tilapia seed

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pangasius catfish (*Pangasianodon hypophthalmus*) has become an important fish for national food security in many Asian countries, including Indonesia. This species has been widely cultivated because of its fast growth and is highly desirable in the European market[1]. Besides, the pangasius catfish is the most popular because of white flesh, contains easily digestible protein, many valuable nutrients and this fish also constitutes a good source of long-chain polyunsaturated fatty acids[2]. Most of the fatty acids in catfish are Monounsaturated fatty acid (MUFA) was the most predominant fatty acid found in all tissues, ranging from 32.7-39.9% and Polyunsaturated fatty acids (PUFA) content was the lowest, ranging from 14.8-24.0%. The fatty acids unsaturated are the most beneficial for human health[3]. Therefore, pangasius catfish has become a commodity with high economic value.

Tra catfish *Pangasianodon hypophthalmus* were imported from Thailand to Indonesia in 1972, only once and were cultivated in Indonesia until now by Hardjamulia[4]. Tra catfish *Pangasianodon hypophthalmus* and Basa catfish *P. bocourti* are two species of nine species of catfish in Vietnam, which cultured. Catfish pangasius a native of the Mekong River in Vietnam has been introduced to many Asian countries. This fish is largely cultured both in monoculture as well as in polyculture. The intensive culture system is putting a lot of stress on catfish pangasius and subsequently, the fish has become susceptible to disease and parasites[1]. Pangasius catfish, as freshwater scaleless fish are very susceptible to be infected ectoparasites and may occur mortality up to 100%[5].

Ichthyophthirius multifiliis, known as "Ich" is an obligate parasite of freshwater fish causing Ichthyophthiriasis. The disease is typically a warm-water disease. A common temperature for Ich outbreaks is ranging 15-25°C[6]. It leads to high mortality and heavy economic loss for cultivated fishes. The development of the parasite is highly dependent on temperature, which is the most essential abiotic factor that has an impact on the whole life cycle of *I. multifiliis*. At warm temperatures (24-26°C) the life cycle is completed in about 48 hrs. Parasites, which live at lower temperatures, produced larger eggs size with higher energy content but exhibited production of eggs are relatively slower. The important role of large egg size as sourcing energy reserves, which are egg membrane-bound lipids that would likely facilitate larval longevity and development success. In contrast, at the higher temperature (ex. 30 degrees), parasites had a higher rate of egg production, sexual maturity faster, the reproductively active period is shorter and containing higher saturated fatty acids compared with polyunsaturated fatty acids. A fatty acid is very important for the metabolic regulation of cell membrane fluidity, which is necessary for larvae to survive warm conditions[7]. The temperature variation affects some parasitic. Most of the parasitic infections were found between November and April when the temperature range varies from 19-26ºC. Therefore, the lower temperature elicits the parasitic growth in fishes meanwhile, the higher temperature retards the growth. The prevalence of the parasites is very much temperature-dependent because most of the parasitic infections occurred during the winter[8]. Parasitic communities in fish vary widely, where the water temperature factor is related to diseases outbreaks[9].

Infection of *Ichthyophthirius multifiliis* in freshwater fish occurring in both temperate and tropical regions throughout the world. The infection causes an extensive economic loss to the aquaculture industry[10]. Currently, fish farms use large amounts of harmful chemicals to control the parasite. This chemical should never be used to treat fish because it can environmentally damage. Presently there is no chemotherapeutic and no commercial vaccine available against the parasite to treat Ich effectively and economically[11]. From 1980 onwards, Picon-Camacho[12] have provided a detailed list of 116 compounds used to control Ich under laboratory or field conditions.

Betel leaf contains phenol active substances and their derivatives, which can inhibit microbial growth. Allylpyrocatechol, a major phenolic constituent of betel leaf, shows activities anti-bacterial effect[13]. Therefore, it suspected phenol compound and distinctive aroma of betel leaf can kill bacteria greater than the phenol group in general. The phenolic-OH group of the betel leaf can cause cell membrane lysis and cause death in ectoparasites[14]. Other research has also proven that the effect of ethanol extract of betel leaves against *Streptococcus agalactiae* infection in Nile Tilapia exhibited the strongest antibacterial activity (0.15-0.60 mg mL^{-1}). It seems that ethanol extract of *Piper betle* can be used promising for sustainable disease management in aquaculture[15]. While administration of the Piper betel leaf extract on pellet feed (20% of *Piper betle* leaf extract/1 kg feed) shows the decrease of intensity of *Trichodina* sp., which infected tilapia (16.17±7.76)[16].

Based on this background, the present study was aimed to check the effect extract of betel leaf against the ectoparasite, *Ichthyophthirius multifiliis* in pangasius catfish in an eco-friendly manner. In the future, betel leaf extract can use extensively to control the disease ectoparasite Ich infection in pangasius catfish.

MATERIALS AND METHODS

Animal sample: The present study was done from March-August, 2020 and carried out at the quality control lab of Freshwater Aquaculture and Fisheries Extension, Bogor, Indonesia. One hundred twenty (120 ind) infected Pangasius catfish came from Research Institute for Freshwater Aquaculture and Fisheries Extension, Bogor, Indonesia. Pangasius catfish were randomly collected in a container. The fish were acclimatized for seven days in tanks containing 500 litres of water, which was aerated with an aerator. The catfish was administered commercial feed, in the morning and evening.10% of the total infected fishes were examined to ensure the existence of ectoparasites. A smear of mucous of the body surface and tail fin were prepared on the clean slide, with drop distilled water and air dried. The air-dried smears were stained with Giemsa solution[10,17]. The average body weight of 4.17±0.96 g and a length of 8.5±0.67 cm were chosen to observe ectoparasites infection. Infected Pangasius catfish transferred and equally distributed to the tank (20 L water) which previously had been administrated with betel leaf extract for 24 hrs, 3 days, at doses 2.5, 5 and 7.5 g L^{-1}. Similar stocking densities were also maintained for control in triplicate. The experimental animals were divided into two groups. The first group for calculating the number of ectoparasites that infect Pangasius catfish (72 individuals) and the second group for calculating the prevalence value (48 individuals).

Extract of the betel leaf: The betel leaves are washed and dried at room temperature, without direct sunlight. Drying using an oven at 40°C and mashed, filtered to obtain a powder. Long-term storage at 26°C in closed containers. The powder of the betel leaf extract is wrapped in gauze and soaked in a treatment tank containing 20L of water for 24 hrs (12 tanks). After 24 hrs, the infected Pangasius catfish was equally distributed I n the tank, 10 individuals per tank, was marked as day zero (H_0). The Observations of ectoparasite infection in Pangasius catfish were carried out on the first day (H_1), the second day (H_2) and the third day (H_3) after soaked. Prevalence value was also carried out referring to the parasite identification method in the Parasitology Laboratory of Research Installation for Fish Disease Control–Research Institute for Freshwater Aquaculture and Fisheries Extension. The prevalence of ectoparasites, temperature, pH, Dissolved Oxygen (DO) levels were calculated.

Data analysis: The number of *Ichthyophthirius multifiliis* in pangasius catfish was analyzed using one-way analysis of variance, $\alpha<0.05$, with the software program SPSS 25 and continued with Duncan's test. The calculation of the prevalence of ectoparasites uses the following Eq.[18]:

$$\text{Prevalence (\%)} = \frac{N}{n} \times 100$$

where, N is the number of samples infected with parasites and n is the number of samples observed.

RESULTS

In this study, the presence of ectoparasite Ich was identified in Pangasius catfish (Fig. 1). The morphology of *Ichthyophthirius multifiliis* has appeared with a large C-shaped macronucleus or horseshoe-shaped macronucleus. The number of ectoparasites in the mucus of the body surface significantly is greater than the number of ectoparasites in the fins (Table 1). The behaviour changes of infected Pangasius catfish showed clinical signs, which always rubbed their body against the side of the tank and swam rapidly. Also, other indicators of infected Pangasius catfish show a white spot all over the body surface and there was a lot of mucus.

The mean score of the number of *Ichthyophthirius multifiliis* in catfish showed a significant difference between treatment and control groups ($p<0.05$). Administrated of betel leaf extract affected the number of *Ichthyophthirius multifiliis* in the treatment group at a dose of 2.5 g L^{-1}, which is the mean score of *Ichthyophthirius multifiliis* in the mucus dorsal was 395.83±10.13 and in mucus fins 145.5±6.84, both are categorized as a very severe infection. In the treatment group at a dose of 5 g L^{-1}, the mean score of the number of *Ichthyophthirius multifiliis* in the mucus dorsal was 172.67±12.17 categorized as a very severe infection, while the mucus fins 68.166±4.54 was categorized as a severe infection. The mean score in the treatment group with a dose of 7.5 g L^{-1} was lower than the two previous groups, which in the mucus dorsal was 48.17±21.46 and the mucus fins were 19.83±3.85, both are categorized as moderate. The dose of 7.5 g L^{-1} of the betel leaf extract is the most optimal because it statistically significantly reduces the number of *Ichthyophthirius multifiliis* in Pangasius catfish. The data in Table 1 was supported by the microscopic observation data of *Ichthyophthirius multifiliis*. On the first day (H_1), it was shown that the number of *Ichthyophthirius multifiliis* in the mucus

Fig. 1: Morphology of *Ichthyophthirius multifiliis* appeared with a large C-shaped macronucleus (magnification40x)

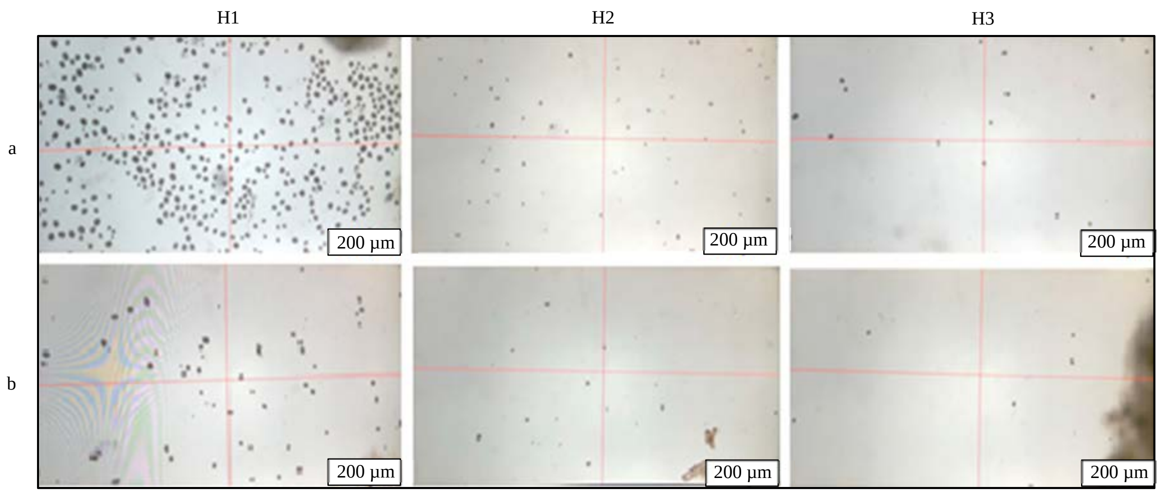

Fig. 2(a-b): Distribution of *Ichthyophthirius multifiliis* in Pangasius catfish (*Pangasianodon hypophthalmus*) in the treatment group after administration of betel leaf extract at a dose of 7.5 g L^{-1}
(a) Mucus and (b) Fins. H1: First day, H2: Second day and H3: Third day (H3)

Table 1: Intensity of *Ichthyophthirius multifiliis* in pangasius catfish

Treatment group (g L^{-1})	fish	Area	Number of ectoparasite Ich (Ind)	Indicators
2.5	18	Mucus dorsal	395.830 ± 10.13^{c}	Very severe infection
		Mucus fin	145.500 ± 6.84^{c}	Very severe infection
5	18	Mucus dorsal	172.670 ± 12.17^{b}	Very severe infection
		Mucus fin	68.166 ± 4.54^{b}	Severe infection
7.5	18	Mucus dorsal	48.170 ± 21.46^{a}	Moderate infection
		Mucus fin	19.830 ± 3.85^{a}	Moderate infection
Control	18	Mucus dorsal	1785.330 ± 19.31^{d}	Super infection
		Mucus fin	638.830 ± 12.72^{d}	Super infection

$^{a-d}$Significant (p<0.05)

and fins of the pangasius catfish had undergone progressively decreased until the second days (H_2) and third days (H_3) (Fig. 2a-b).

The prevalence of ectoparasites in fish can be seen from how many ectoparasites were identified in the laboratory and the severity of the fish organs infected with parasites (Table 2). The prevalence value of *Ichthyophthirius multifiliis* in all treatment groups was 100%. Besides these susceptibility factors, water quality factors also support the prevalence value. Water quality parameters, temperature, power of hydrogen (pH) and Dissolved Oxygen (DO) can be seen in Table 3. The temperature in the treatment tanks ranges from

Table 2: Prevalence of *Ichthyophthirius multifiliis* infecting pangasius catfish (*Pangasius hypophthalmus*)

Treatment group (g L^{-1})	Observed fish (n)	Infected catfish (N)	Area	Prevalence (%)
2.5	12	12	Mucus dorsal	100
			Mucus fin	100
5	12	12	Mucus dorsal	100
			Mucus fin	100
7.5	12	12	Mucus dorsal	100
			Mucus fin	100
Control	12	12	Mucus dorsal	100
			Mucus fin	100

N: Number of infected fish (ind) and n: Number of samples observed (ind)

Table 3: Water quality parameters of the tanks

Treatment group (g L^{-1})	pH			Temperature (°C)			Dissolved oxygen		
	H_1	H_2	H_3	H_1	H_2	H_3	H_1	H_2	H_3
2.5	7.53	7.53	7.54	27.37	27.50	27.40	5.50	5.63	5.62
5	7.53	7.48	7.49	27.43	27.43	27.40	6.63	6.61	6.60
7.5	7.56	7.55	7.60	27.83	27.83	27.83	5.60	5.77	5.76
Control	7.55	7.55	7.54	27.57	27.63	27.63	7.33	7.32	7.34

H_1: First day, H_2: Second day and H_3: Third day

27.37-27.83°C, pH 7.48-7.60 and DO 5.5-7.3. The water quality is suitable for the life cycle of *Ichthyophthirius multifiliis* and also for the survival of catfish.

DISCUSSION

The *Ichthyophthirius multifiliis*, a ciliate pathogen was identified in this study, as the causative agent of white spot diseases. The outbreak (prevalence 100%) of white spot disease resulting from *Ichthyophthirius multifiliis* infection might be associated with water temperature. Infected Pangasius catfish showed several clinical signs, swimming rapidly and rubbing their bodies against the sides of the tanks. The research supported by Mamun[11] found salt-like granule white spots on the body surface of striped catfish. The present study shows that the ectoparasite intensity in the mucus on the dorsal surface of the body is significantly greater than the ectoparasite intensity in the mucus of the caudal fin. The fish mucus is considered as one of the important components of the first line of defence against infectious pathogens. Fish mucus is considered as one of the important components of the first line of defence against infectious pathogens. Mucus is a suitable viscous colloid for the growth of ectoparasites and also as a food source. The caudal and anal fins contain not too much mucus because the hard fin structure makes it difficult for the ectoparasites to attach to the fins[19]. In general, the dorsal of the body in *Pangasianodon hypophthalmus* had more mucous cells than the ventral of the body and the highest mucous cells count was in the tail region[20]. Increased mucus production is considered as an adaptation for protection, assisting the fish to adjust to the changing environment, especially in fish without scale. Therefore, the scales fish rely on both external and internal mucus production to protect themselves against the infectious agents and the toxins produced by the predators. The other function of mucus is as a barrier, which is as a protection against the surrounding environment with biotic and abiotic factors.

The mucus surfaces are dynamic matrices, their composition varies influenced by various endogenous (sex and developmental stage) and exogenous factors (such as infection). The research of Rajan[21] shows that fish tend to increase their mucus secretion in stress conditions and also can change their composition of mucus when infected by ectoparasites. The mucus secretion contributes to the defence against these pathogens, sometimes excess mucus secretion can facilitate the increase of pathogenic bacteria[22]. In fish undergone stress, usually have a high number of proteins on their mucus, a gel-like material is secreted by the epidermis[23]. Mucus is constantly secreted and replaced in most fishes, this is to prevent the invasion of infectious microorganisms and metazoan parasites. The mucus is the food sources of the parasite and also the role as a defence, which is known as the mucosal immune systems. The process of the infection by ectoparasite Ich is started in the mucus surface, which is the first-line defence against the encounter. Epidermal goblet cells secrete the external mucous gel, as a layer of adherent mucus that covers the living epithelial cells. Water and glycoproteins are the main components of mucus external, which is containing mucin. Mucin acts as a filter for pathogens and prevents the pathogen to attach to the underlying tissues[23]. Based on the results of the current study, it suggests that increased intensity of the ectoparasite Ich in the mucus of the dorsal surface body was caused by more mucous cells in this region. The exposure of ectoparasite Ich infection to Pangasius catfish in the water was potentially high accrue in the dorsal segment of the body surface. It becomes a reason that there is the increased intensity of the ectoparasites in this region.

The investigations presented here indicate the prevalence value of infected Pangasius catfish both the mucus of dorsal body surface and the mucus fin is 100%. Although, the amount of ectoparasites in the mucus of dorsal the body surface is higher than the number of ectoparasites in the mucus fin. The criteria of parasite infection in all the Pangasius catfish were a very severe infection and the attack rate was "always". This value is very high, possibly due to the density of catfish in the aquaculture ponds and the process of contact between the fish allowing the spread of ectoparasites Ich. Parasites can infect fish directly or indirectly. Direct infection, through direct contact between fish, while indirect infection through decreased immunity of fish due to stress, so that parasites easily infect fish[24].

The high prevalence value in this study shows that Pangasius catfish is susceptible to attack by *Ichthyophthirius multifiliis*, as evidenced by all fish treatment and control groups were infected with ectoparasites. Research by Xu[25], catfish Ich-infected showed significantly higher mortality (91.7%). It means the catfish is very susceptible. The prevalence value of *Ichthyophthirius multifiliis* in *Pangasianodon hypophthalmus* is 100%[11], cyprinid and salmonid were infested *Ichthyophthirius multifiliis* (28.2%)[26]. Differences in prevalence values are related to species susceptibility and differences in fish morphology. Freshwater fish, without scales, are more susceptible to ectoparasite and monogenic infections than scaly fish[6]. The fish can mount protective immune responses against various parasites. The defence mechanisms are responsible for the protection of fish against infections with these ectoparasites. The mucus on the surface of the body in Infected fish parasites, was secreted as a defence against ectoparasites infections[27].

The high infection can also be caused by the stage of development of the ectoparasite, which infected Pangasius catfish. In this study, it was suspected that the ectoparasite Ich was in the tomont phase. This life cycle of the ectoparasites is highly dependent on water temperature, wherever the lower temperature elicits the parasitic growth in fishes meanwhile, the higher temperature retards the growth[8]. In the current study, it was suspected that the life cycle of tomont was longer in the water temperature below 37°C than the life cycle of tomont at approximately 37°C. The Parasites, which live at lower temperatures, would likely facilitate larval longevity and development success[7] and most of the parasitic infections were found between the temperature range varies from 19-26°C. It becomes the reason that administration of betel leaf extract at a dose of 7.5 g L^{-1} does not kill ectoparasite as wholly. The reproductive potential of tomonts could be related to whether the tomont has finished developing the cyst wall. The tomont attaches to the bottom of substrates or plants and begins to undergo mitosis (binary fission). It is believed that cyst plays an important role in protection[28]. The cyst as a sticky capsule consists of a translucent proteinaceous that is secreted by tomont. The tomont can attach to the substrate via a cyst, that cannot be penetrated by drugs[29]. In the life cycle of Ich, tomont phase is more resistant and less susceptible to betel leaf extract than the infective phase (theront), because the tomont is protected by cysts and mucus. In this study, administration of betel leaf extract at a dose of 2.5, 5 and 7.5 g L^{-1} for 3 days causes a decrease in the number of ectoparasites Ich, which infected the Pangasius catfish, even though some ectoparasites still infect Pangasius catfish.

This research is also supported by Syahidah[30] that hydroxychavicol and eugenol isolated from *Piper betle* leaves were responsible for the antibacterial activity. The hydroxychavicol as a phenolic compound maybe also have antiparasitic activity. Hydroxychavicol can bacterial cell death by ROS generation and cause DNA damage[31]. Research by Agustina and Layoo[16] also found that the administration of betel leaf extract solution reduced significantly *Trichodina* sp. infection in tilapia (*Oreochromis niloticus*). A study *in vitro* by Saptiani[32] showed that the ethanol extract of *P. betles* at a concentration of 1000 ppm is an optimum concentration for inhibiting the microbe growth aquatic. In the current study, betel leaf extract significantly decreased the amount of ectoparasite Ich in both dorsal and fin mucus, which was seen on microscopic observations after the third day (H_3) in each treatment. The contains of phenol from the *P. betle* leaves and its derivatives, such as chavicol and eugenol, have a strong pungent aromatic flavor[33]. It suspected the aroma of the leaves of the *P. betle* leaves caused by eugenol content and the powerful antiseptic properties of chavicol is significantly decreased the amount of ectoparasite Ich. The high phenol content of the aromatic volatile oil from betel leaves (82.8%) causes the distinctive aroma of betel leaf[19]. The phenolic group (OH) on the aromatic ring structure contribute to penetration to the cell plasma membrane and cause damage to the plasma membrane[14].

A decrease of number in the ectoparasite of Ich was also found by increasing the concentration of flavonoids in the leaf betel extract. It is assumed that the phenol content in the betel leaf extract at dose 7.5 g L^{-1} as the optimal dose, has damaged the ectoparasite plasma and cause the ectoparasites death. The presence of phenolic hydroxyl groups can easily penetrate the cell membrane, cause cell damage. this result in inhibition of cleavage of tomont and the release of theronts[34]. Disruption of the transport of ions in the cell membrane can inhibit ectoparasite growth and cause cell death. For this reason, a higher concentration of this phenolic compound was needed to wholly kill tomonts, because the administration of the betel leaf extract at dose 7.5 g L^{-1} not fully yet inhibit ectoparasites Ich and some ectoparasites still infect Pangasius catfish. Therefore, needed the betel leaf extract with a higher dose. The high concentrations of the betel leaf extract maybe have also a high presence of phenolic groups, so can easily penetrate the cell membrane to inhibit tomont division. A longer exposure time of a natural bioactive compound shows that the compound may be more efficient to control the ectoparasites infection. This is due to the phenolic compound has low toxicity and also and is a safe agent to control ectoparasites infection on zebrafish in the aquaculture industry[35]. Therefore, the betel leaf extract as a natural bioactive compound, which is readily available and affordable demonstrates the application potential as a therapeutic agent for ectoparasite infections.

CONCLUSION

It can be concluded that the leaf betel extracts affect a decrease significantly the number of ectoparasites *Ichthyophthirius multifiliis* in Pangasius catfish (*Pangasianodon hypophthalmus*). A dose of 7.5 g L^{-1} is as optimum dose, although some ectoparasites still infect Pangasius catfish. In future research, the betel leaf extract dosage needs to be increased so that it is more efficient and with a longer soaking time.

SIGNIFICANCE STATEMENT

The use of *P. betle* leaves for ectoparasite control is an environmentally eco-friendly technology concept. The potential of *P. betle* leaves in this research was proved to control ectoparasite infection Ich and can even minimize the mortality of catfish seeds. This study will help the researcher to uncover the use of betel leaf, which is found in almost all regions in Indonesia that many researchers were not able to explore. The use of *P. betle* leaves as a natural herb which relatively cheap, its low toxicity and also safe against pangasius catfish, become the considering in the future.

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