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RESEARCH PAPER

Evaluation of the Effects of *Piper betle* Supplementation as a Natural Antibiotic Growth Promoter (NAGP) on Antimicrobial Activity, Feed Acceptance and Growth Performance of Kelah (*Tor* sp.) in a Tank System

Hanan Mohd Yusof[1,4], Ahmad Baihaqi Othman[1], Nik Haiha Nik Yusoff[2], Muhd_Zudaidy Jaapar[1,4], Azila Abdullah[3], M. Firdaus Nawi[4], Irfan-Hakimi, R.[4] and Nur-Nazifah, M.[4*]

[1]Freshwater Fisheries Research Division, FRI Glami Lemi, Titi, Jelebu, Negeri Sembilan, Malaysia
[2]Marine Finfish Research Division, FRI Tg Demong, Besut, Terengganu, Malaysia
[3]National Fish Health, Institut Penyelidikan Perikanan, Batu Maung, Penang, Malaysia
[4]Kuliyyah of Science, International Islamic University, Malaysia, Kuantan, Pahang, Malaysia

*Corresponding author: nurnazifah@iium.edu.my (**ORCID ID:** 0000-0003-2160-4754)

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ABSTRACT

The negative side-effects of the usage of synthetic antibiotic growth promoters (AGP) in feeds for animals and humans have been widely understood, although it positively promotes growth and gives better economic returns to the farmers. Thus, the present study was conducted to evaluate the potential of *Piper betle* extract as an herbal product as a feed additive and a natural antibiotic growth promoter (NAGP) toward kelah (*Tor* sp.) on antimicrobial activity, feed acceptance, and growth performance. In this study, for antimicrobial activity, several concentration levels of *Piper betle* extract were tested to observe the optimum dosage for bacterial inactivation of *Aeromonas hydrophila* obtained from the kelah specimen. Then, in evaluating the optimal palatability of *Piper betle* concentration for kelah, the fish were divided into seven treatment groups, one of which was used as a control and daily fed at 3% of the fish's body weight. In the growth study, two treatment methods were used, which were once a month (S1) and fortnightly (S2) feedings of a diet treated with 100 ppm *Piper betle* extract, with a non-treated *Piper betle* extract diet serving as a control (C). All treatments were conducted in duplicate and the fish were stocked at 10 fish m^{-2} in 3MT tanks. Fishes were fed ad libitum, twice daily. The results showed the optimal dosage for *Piper betle* extract was identified at 100 ppm with a significant different ($P > 0.05$) in the size of the *Aeromonas hydrophila* bacterial inhibitory zone, with diameters of 18.7± 0.6 mm. However, there was no significant difference between feed acceptance by kelah on *a Piper betle* treated diet up to 100 ppm dose and a control diet with a value of 1.32 % body weight per feeding session. While at 182 days of growth study, the results show that the best growth performance of kelah according to body weight gain (BWG) was treatment S2 with 131.9 ± 9.1 %, followed by S1 and control at 104.8 ± 10.2 % and 84.2 ± 9.5 %, with a significant difference ($P < 0.05$). Additionally, a significant better ($P < 0.05$) specific growth rate (SGR) of S1 and S2 treatments was valued at 0.39 ± 0.10 and 0.47 ± 0.02, respectively, compared to the control treatment at 0.34 ±0.03. While the S2 treatment significantly showed the best feed conversion ratio (FCR) compared to the S1 and control treatments, with 3.57 ±0.17, 4.27 ±1.13, and 5.02 ±0.49, respectively. In overall, there was no significant difference in survival

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rate among all treatments in this study. Thus, the results suggest that *Piper betle* can be considered as a NAGP in the diet at the optimum dose of 100 ppm, with fortnightly application as the best feeding regime for kelah culture.

HIGHLIGHTS

- 100 ppm of Piper betle leaf extract shows the best antibacterial activity against *Aeromonas hydrophila* compared to Oxytetracycline and alcohol.
- Dosage of 100 ppm Piper betle leaf extract incorporated in feed shows good feeding acceptance and promotes high growth performances in kelah.
- Application of Piper betle leaf extract as a natural antibiotic growth promoter (NAGP) additive in feed given fortnightly feeding regime has no negative impact on residues or the risk of bacterial resistance.

Keywords: *Piper betle*, kelah (*Tor* sp.), natural antibiotic growth promoter (NAGP), antibacterial, growth performance

The aquaculture industry is one of the most rapidly expanding industries. Using the previous dynamic prediction model based on production status and worldwide fishing demand from 2000 to 2008, demand is predicted to rise by 20% by 2030, to 186 million tonnes, compared to 2011. Based on demographic trends around the world (The World Bank, 2013), the aquaculture sector accounts for 60% of the target fish demand, satisfying the needs of human beings directly. Based on the rapid development of the aquaculture business in recent years, various problems as well as solution methods connected to illnesses, nutrition, pollution, and genetics must be addressed as quickly as possible. Various methods are used for the purpose of producing productive and cost-effective aquaculture products, such as increasing livestock density, genetic development of breeds, the use of cost-effective diets, and the use of hormones and antibiotics, which certainly have negative consequences not only on livestock and the environment, but also on humans.

In general, the aspects of nutrition play a significant role in guaranteeing the sustainable development of all industries in the aquaculture sector, including in developed and developing nations, because it involves the development of growth, environmental integrity, and animal health. However, the widespread use of synthetic antibiotics and therapeutic chemicals for treatment or prophylaxis, as well as growth stimulants such as antibiotic growth promoters (AGP) in food to achieve fast and high livestock production by farmers, has become a major issue that has been widely opposed. As a result, scientists are striving to discover alternative materials and methods to synthetic antibiotics and therapeutic compounds, such as organic acids, probiotics, prebiotics, and phytobiotics, which are safe for both livestock and humans as well as the material's function as an additive (Castillo-Lopez, *et al.* 2017; Huyghebaert, G. *et al.* 2011; Upadhayay, A. and Upadhyaya, I. 2014). Apart from improving animal health and growth, natural antibiotic growth promoter (NAGP) from medicinal plants material has no negative impact on residues or the risk of bacterial resistance because it biodegradable qualities, availability, and simplicity of cultivation. Therefore, the use of medicinal plants and their derivatives in aquaculture is rising worldwide on a daily basis as continous discoveries of its bioactive compounds that can be employed in sustainable aquaculture as a phytotherapeutic agent.

Tacon *et al.* (2011) predict that global fish feed output will increase by 11.1 percent per year to 70.9 million metric tonnes by 2020, implying a large potential demand for NAGP as an alternative to AGP. Therefore, seeing the great potential to develop natural antibiotics, the Fisheries Research Institute (FRI), Department of Fisheries Malaysia, has pioneered and actively conducted research based on phytobiotics, especially from herbal sources, to replace synthetic antibiotics on the market for aquaculture use. One of the products that has been developed by FRI Tanjung Demong based on herbs is the patented SirehMAX TM (Patent re. no. : MY-176273-A), which is a natural anti-microbial ingredient based on *Piper betle* leaf extract. Several studies have shown that SirehMAX TM products are effective as antibiotics against pathogens that attack aquaculture fish species, causing disease and death as well as economic losses to farmers (Ahmad-Baihaqi *et al.* 2018; Nik-Haiha,

2011). Disease caused by *Aeromonas hydrophila* has also been studied using herbal extract (Mohamad & Abasali, 2010) and found to be effective in cyprinid. Effectiveness of *Piper betle* extract as a treatment and prevention against vibriosis has been previously reported under laboratory environment (Ahmad-Baihaqi *et al.* 2018). Thus, this study is conducted to evaluate the effectiveness of herbal plant, *Piper betle* incorporating in a prepared formulation diet as a medicated feed in controlling *Aeromonas* sp. infection in Kelah (*Tor* sp.). Several observations by researchers have found that aeromonads often infect the Mahseer throughout the culture period (Lau, M.M.L. *et al.* 2021; Kumar, R. *et al.* 2016) and occasionally it is associated with primary infection of *Piscinoodinium* sp. (Rosly, *et al.* 2008).

Kelah (*Tor* sp.) is a riverine cyprinid that is also known as the Malaysian Mahseer. It is one of Malaysia's most expensive freshwater fish. Anglers consider it to be one of the best game fish because of its exquisite taste (Ng, 2004, Asaduzzaman *et al.* 2018). However, because it is a tasty fish, the price is high, ranging from RM 250 to RM 350 (USD 60 to 85) per kilogramme (Azlinda, 2019). As a result, fishing demand on natural resources has increased. Kelah are being intensively farmed by farmers in ponds or tanks due to increased demand. As a result, a Mahseer breeding programme was established in Malaysia in 2015 to accommodate the growing demand for seeds from farmers.

Thus, this study was carried out to evaluate the supplementation of *Piper betle* orally in fish feed as a NAGP additive for kelah, was effective and suitable as an antibacterial agent, feed acceptability and growth promoter. Furthermore, the goal of this research is to determine the optimal dietary regimen for using herbal compound from *Piper betle* as a mean to support sustainable aquaculture practice in kelah farming. As a note, this study was also part of a programme under the 11th Malaysia Plan (RMK-11), which was the development of kelah as a new aquaculture resource species in Malaysia, and this study directly supports the program's efforts.

MATERIALS AND METHODS

Antibacterial activity and optimal dosage for bacterial inactivation

The Bauer *et al.* (1966) method for determining antibacterial activity and optimal dose concentration for *Piper betle* extract is used to guide the modified disc dyeing method. Different concentration levels were generated, including 2.5 ppm, 5.0 ppm, 10 ppm, 20 ppm, 40 ppm, 80 ppm, and 100 ppm. The selection of this dose was based on reference from previous studies by Rahman and Hossain (2010) related effect of antimicrobial activity on *Aeromonas hydrophila* against Oxytetracycline (OTC). Each extract was dipped in various concentrations on sterilised 6 mm disc filter paper and allowed to dry in a sterile atmosphere. The *Aeromonas hydrophila* bacterial cultures were equally dispersed on a petri plate containing sterilised soy trypticase (TSA) media. Three replications of dipping discs with varied concentrations of *Piper betle* extract were made and placed on the prepared petri dishes. Each replication, on the other hand, included a control disc that was dipped in ethanol. All petri dishes were incubated for 24 hours at 37°C in an incubation oven. The diameter of bacterial inactivation was measured and studied.

Feed acceptance of *Piper betle* to fish

The goal of this test is to find out how much *Piper betle* extract was given to the fish. Based on the findings of the optimal dose determination test, seven dose concentrations, which are 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm, 200 ppm, and 300 ppm, were chosen for testing by spraying uniformly on a 32% crude protein commercial Tilapia starter feed (Dindings 920) of 2.0 mm diameter pellet size. Meanwhile, in this study, the feed that had not been sprayed with *Piper betle* extract served as a control. 160 kelah fish with an average weight of 20g were placed in 16 (1 MT) tanks from the same population stock and cohort. The fish were divided into seven treatment groups, including a duplication control group, at a density of 10 m^{-2} each. All of the fish were fed at a rate of 3% of their body weight per day. Throughout the trial, the photoperiod was maintained at 12:12 (light: dark), with light delivered from 07:00 to 18:00. The fish were fed experimental feed two times daily at 09:00 and 16:00 for three days. Each experimental feed was hand-fed for ten minutes, or until visual satiation was achieved. If any feed residues remained, they were collected, dried, and weighed for actual feed intake. The findings using modified method by Caimi *et al.*

(2020) are expressed in terms of fish body weight using the formula provided below:

Feed acceptance (% of body weight/feeding session) = (feed intake (g/session)/biomass of fish in the tank) × 100

Evaluation of the growth performance of fish with different feeding regimes

The treatment diet in this study was sprayed with *Piper betle* extract based on the selected dose of concentration test results and the best acceptance test completed earlier. Then, there were two treatment strategies based on distinct dietary regimes, namely fish feeding with the *Piper betle* treatment diet once a month (S1) and fortnightly (S2), with a control diet of untreated *Piper betle* extract (C). All treatments were duplicated in a 3 MT cement tank with a fish density of 15 fish per m^2 during a 182-day test period. The test fish were fed routinely with commercial Tilapia starter feed (Dindings 920) at a rate of 5% to 3% of their body weight. Throughout the test period, any nutritional activity responses and health levels of the fish were evaluated and documented. The body weight gain (BWG) and the specific growth rate (SGR) of the test fish will be determined. Meanwhile, when the study is completed on the 182nd day, other parameters such as the feed conversion rate (FCR), mortality rate, and ultimate weight growth will be obtained.

Growth performance

At the end of the feeding experiment, all fish were weighted to calculate the initial weight (Wi),final weight (Wf), body weight gain (BWG), specific growth rate (SGR), and feed conversion ratio (FCR). Also, Nf and Ni represent the initial and final numbers of fish to calculate survival rate (SR). Calculations were conducted using the following formula:

$$BWG\ (\%) = (Wf - Wi)/Wi \times 100$$

$$SGR\ (\%) = (\ln Wf - \ln Wi)/t \times 100,$$

where t was the duration of the experimental period (days)

$$FCR = Feed\ consumed\ (g)/(Wf - Wi)$$

$$SR\ (\%) = (Nf - Ni)\ \ 100$$

Statistical analysis

All data collected from the study will be subjected to a one-way variance (ANOVA), and the difference between means will be tested by Duncan's multiple range test for means comparison between groups using SPSS software version 23 (SPSS Inc. Chicago IL. USA). All the quantitative analyses were expressed as mean ± standard deviation (SD) for duplications. When P <0.05, the differences were considered statistically significant.

RESULTS

The result of antibacterial studyhas shown that the best dose of *Piper betle* extract was 100 ppm with a significant difference (P<0.05) compared to other concentration level. As shown in Fig. 1, the diameter of the *Aeromonas hydrophila* bacterial inhibition zone of different *Piper betle* extract concentration ranges from 3.0 ± 1.0 mm to 18.7 ± 0.6 mm.

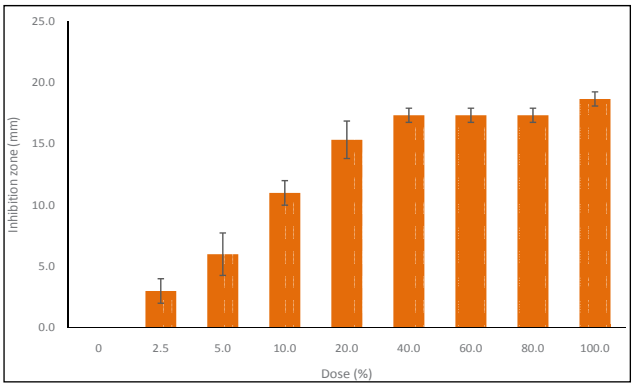

Fig. 1: Determination of the optimum *Piper betle* extract dose for antibacterial activity for *Aeromonas hydrophilla*

The determination of the optimal concentration of *Piper betel* extract in feed for kelah is shown in figure 2. The acceptable dose concentration of *Piper betel* extract by kelah is determined to be between 20 ppm and 100 ppm. However, a dose of 100 ppm was chosen because its antimicrobial action was significantly (P<0.05) more effective than other concentration doses. Although, at the dosage of 20 ppm demonstrated a significant (P<0.05) highest feed acceptance of 1.52 ± 0.03(% BW/ feeding session) compared to other treatments, but, unfortunately it only gave a moderate antibacterial action. Meanwhile, as for 200 and 300 ppm doses of *Piper betel* extract it demonstrated a significant rejection of feed by kelah (P< 0.05) at feed acceptance value of 1.09 ± 0.08 and 1.13 ± 0.06 (% BW/ feeding

session) respectively.

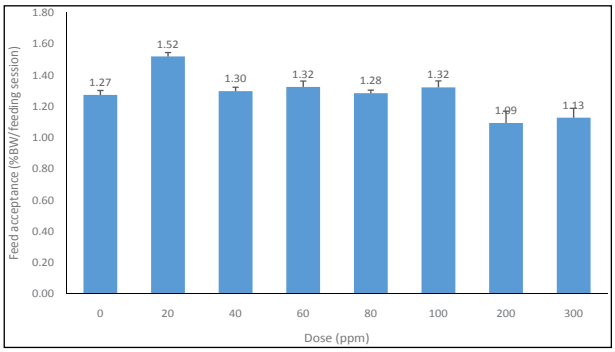

Fig. 2: Feed acceptance of kelah fed with treatment diet of different *Piper betle* extract dose concentrations.

On the 182nd day of the study period and referring to table 1, the results indicate that the treatment S2 had the highest growth performance in terms of body weight gain (BWG) at 131.9 ± 9.1%, followed by S1 and control at 104.8 ±10.2% and 84.2 ±9.5 % respectively, with a significant difference ($P < 0.05$). Additionally, the S1 and S2 diets had a significantly higher ($P > 0.05$) specific growth rate (SGR), 0.39 ± 0.10 % $day^{-1}$and 0.47 ±0.02 %day^{-1}, respectively, as compared to the control diet, which had an SGR of 0.34 ±0.03. While the S2 diet significantly showed the best feed conversion ratio (FCR) compared to the S1 and control diet, with 3.57 ± 0.17, 4.27 ± 1.13, and 5.02 ± 0.49, respectively. However, no significant difference in survival rates was seen in this evaluation study among all diets.

Table 1: Growth performance of Kelah fed with different feeding regime using Piper betle extract treated diet

Parameters	C	S1	S2
Initial weight (Wi) (g)	28.00 ± 1.47^{a}	26.69 ± 4.03^{a}	26.76 ± 0.08^{a}
Final weight (Wf) (g)	51.51 ± 0.03^{a}	53.97 ± 1.11^{a}	62.05 ± 2.61^{b}
Body weight gain (BWG) (%)	84.25 ± 9.53^{a}	104.81 ± 9.04^{b}	131.87 ± 9.05^{c}
Survival rate (SR) (%)	96.65 ± 4.74^{a}	98.35 ± 2.33^{a}	96.65 ± 4.74^{a}
Specific growth rate (SGR) (%day^{-1})	0.34 ± 0.03^{a}	0.39 ± 0.10^{ab}	0.47 ± 0.02^{b}
Feed conversion ratio (FCR)	5.02 ± 0.49^{a}	4.27 ± 1.13^{ab}	3.57 ± 0.17^{b}

Data are the mean ±SD of duplicates for each treatment group. Different alphabet letters in same column after each growth performances and survival rate value indicate significant differences in each column group ($P<0.05$).

DISCUSSION

Several herbal or plant derivatives have been previously identified for their potential as antibacterial, antiparasitic, immunostimulant & growth promoter (Ramudu & Dash, 2013). Throughout the study, there were no side effects or severe mortality in experimental fish. High survival rate of more than 95 % in all diets was also observed, indicating that a diet treated with plant based source of *Piper betle* extract was safe and suitable as an alternative to synthetic AGP and can be termed as natural antibiotic growth promoter (NAGP). This is supported by previous studies proving that plant extract materials are non-toxic as well as safe (Subeena, B. and Navaraj, P.S. 2012; Zakaria, Z.A. *et al.* 2011). Traditionally, farmers have been using oxytetracycline, which is one of the few chemotherapeutic agents approved by the USFDA for the treatment of certain bacterial infections in medium- and warm-water fin fish (Julinta *et al.* 2017). However, recent findings demonstrated the effects of antibiotic resistance on pathogens in the aquaculture industry, such as salmon (Miranda *et al.* 2018). There are several antibiotics in the aquaculture sector that have been banned in most countries as they have caused resistance to pathogenic bacteria and also have carcinogenic effects as well as mutations detrimental to human health and the environment, such as Nitrofuran and Chlorofenicol (Schnick, 1991). Consequently, to minimize the loss in aquaculture production, there is a need to find alternatives solution using herbal plants for aquatic animal health management. According to Madhuri *et al.* (2012), plant is an excellent alternative to be used to solve bacterial infection as it is environmental friendly and cheap.

Julianta *et al.* (2017) have previously reported that the efficiency of *Piper betel* extract as an antibacterial agent against pathogenic *Aeromonas hydrophila* was comparable to that of commercial antibiotics such as oxytetracycline and gentamicin, with an inhibition zone of 18.0 mm diameter. Interestingly, Mahmoud *et al.* (2017) discovered that curcumin (*Curcuma longa*), one of the most effective natural antibacterial agents, can only inhibit a smaller region of inhibition, 14.55 mm in diameter, in a similar study. According to Farnsworth and Bunyapraphatsara (1992), the antibacterial action

of *Piper betel* leaf is thought to be related to the presence of phenolic compounds.

The feed acceptance data is similarly close to those reported in previous research by Saufinas *et al.* (2013), which placed the daily feed requirement for kelah (*Tor tambroides)* at 3% of BWof feed / day. Whereas, it was discovered in this study that kelah consumed up to 2.64 % BW of feed / day or an average of 1.32 % BW / feeding session on a 100 ppm *Piper betle* treatment diet. There are several studies also that have been conducted on fish and poultry that can improve the appetite of livestock (Augustina, 2019). According to Kawamura *et al.* (2019), the active chemicals in certain plants extract demonstrated the potential to promote appetite and feed consumption by an animal by triggering the release of digestive enzymes.

A significant improvement on final weight, BWG and SGR for kelah fed on NAGP diet treated with 100 ppm *Piper betle,* when applied according to S2 regime than S1 regime and control treatment. Meanwhile, it was also showed in the present study, thatS2 treatment gave a significant better feed conversion ratio (FCR) compared to the control diet. This finding alsoindicates the same trend as several previous studies using oxytetra cycline as AGP by Reda, *et al.* (2013) on Nile Tilapia (*Orechromis niloticus*); Sanchez-Martinez *et al.* (2008) on Channel Catfish (*Ictalurus punctatus*). However, a conflict finding by Trushenski, *et al.* (2018) was reported regarding oxytetracycline administration, that had no effect on the survival or growth of Channel Catfish (*Ictalurus punctatus*), hybrid Striped Bass, Nile Tilapia, (*Oreochromis niloticus*), and Rainbow Trout, with no significant differences in weight gain, feed conversion ratio, or specific growth rate.

The results of the study also showed similar SGR and FCR values for kelah fed with100 ppm of *Piper betle* extract feed according to S2 feeding regime, compared to the previous study by Saufinas (2019), although the kelah was fed with high protein diet. This is probably due to its biologically slow growth performance of kelah compared to most other freshwater fish species as been reported by many researchers (Asaduzzaman *et al.* 2018; Rosly *et al.* 2006; Igram *et al.* 2007).

In general, the concept of application of antibiotics on growth promotion mechanisms in animal had been summarized by Reda *et al.* (2013), which are through reducing subclinical infections, reducing the generation of harmful bacterial metabolites in the intestines, sparing bacteria nutrient use, and increasing absorptive efficiency through intestinal wall thinning. Another opinion, according to Maheshappa *et al.* (1999), herbal active ingredients in fish diet are able to stimulate the production of digestive enzymes, in turn it can stimulate appetite and indirectly increase dietary intake in addition to making livestock individuals healthier. In addition, the nature of NAGP material from herbal sources is able to stimulate transcription and in turn lead to effective digestion of high protein material (Citarasu, T. 2010).

CONCLUSION

The application of *Piper betle* leaf extract as a natural antibiotic growth promoter (NAGP) additive in feed for kelah in the current study has revealed that the optimal dosage was at 100 ppm has resulted with good feeding acceptance, best antibacterial activity and also promotes growth performance. Besides, this NAGP is best to be applied according to fortnightly feeding regime. Overall, NAGP has no negative impact on residues or the risk of bacterial resistance but also promotes sustainable aquaculture.

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