คุณสมบัติในการยับยั้งเชื้อ และประสิทธิภาพของใบหม่อนในการเป็นอาหารเสริมต่อการเจริญเติบโตและความต้านทานเชื้อ *Aeromonas hydrophila* ในปลานิล

Antibacterial Property and Potential use Mulberry leaves (*Morus alba* Linn.) as Dietary Supplement on Growth Performance and Disease Resistance against *Aeromonas hydrophila* in Nile Tilapia (*Oreochromis niloticus*)

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บทคัดย่อ

การศึกษาครั้งนี้เป็นการศึกษาประสิทธิภาพของสารสกัดใบหม่อนในการต้านเชื้อแบคทีเรีย *Aeromanas hydrophila* และประสิทธิภาพของใบหม่อนต่อการเจริญเติบโตและความต้านทานเชื้อ *A. hydrophila* ในปลานิล โดยใช้แอลกอฮอลล์ความเข้มข้น 95% เป็นตัวทำละลายในการสกัดใบหม่อน ผลการทดสอบประสิทธิภาพของสารสกัดใบหม่อน พบว่า ขนาดเส้นผ่านศูนย์กลางของบริเวณที่มีการยับยั้งเชื้อแบคทีเรีย (inhibition zone) เท่ากับ 10.77±0.25 มิลลิเมตร และมีค่าความเข้มข้นต่ำสุดในการยับยั้งเชื้อแบคทีเรีย (MIC) เท่ากับ 2,785 มิลลิกรัมต่อลิตร แต่ไม่พบระดับความเข้มข้นใดที่สามารถฆ่าเชื้อแบคทีเรีย (MBC) ได้ นอกจากนี้ ทำการทดลองโดยนำใบหม่อนผสมกับอาหารสูตรผสมในอัตราที่แตกต่างกัน 4 ระดับ คือ 0, 5, 10 และ 15 เปอร์เซ็นต์ จากนั้นนำมาเลี้ยงปลานิลขนาดเริ่มต้นที่น้ำหนัก 2.34±0.82 กรัม และ ความยาว 5.56±0.23 เซนติเมตร โดยให้อาหารกินจนอิ่ม วันละ 2 ครั้ง เป็นเวลา 8 สัปดาห์ ผลการทดลองพบว่า อัตราการเจริญเติบโตจำเพาะ อัตราการแลกเนื้อ ประสิทธิภาพการใช้อาหาร อัตราการรอดตาย อัตราส่วนประ สิทธิภาพของโปรตีน และค่าดัชนีตับในทุกชุดการทดลองไม่มีความแตกต่างกันทางสถิติ ($P>0.05$) นอกจากนี้ผลการทดลองต่อความต้านทานเชื้อ *A.hydrophila* พบว่าปลาที่ได้รับอาหารผสมใบหม่อน 15 % มีอัตราการตายต่ำสุดแตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างชุดการทดลอง ($P<0.05$) และมีเปอร์เซ็นต์การรอดตายสัมพัทธ์ของปลา (RPS) สูงสุด

คำสำคัญ: ใบหม่อน ปลานิล *Aeromonas hydrophila* เปอร์เซ็นต์การรอดตายสัมพัทธ์

Abstract

The study on antibacterial activity of mulberry (*Morus alba* Linn.) extract against *Aeromonas hydrophila* and effect of supplementing diet with mulberry leaves on growth performance and disease resistance against *A. hydrophila* in Nile tilapia (*Oreochromis niloticus*) were conduct. Mulberry leaves were extracted by ethanol 95%. The result of antibacterial screening performed by disc method was 10.77±0.25 mm in diameter of inhibition zone. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentrations (MBC) of mulberry extract were also determined by broth dilution method. The results showed that MIC was 2785 ppm and MBC was not observed in any concentration of mulberry extracts. Moreover, the experimental fish with an initial mean weight of 2.34±0.82 g and length of 5.56±0.23 cm were stocked in 12 glasses aquariums. Each aquarium has dimension 45×90×45 cm and was filled with 150 liters of water at a stocking density of 20 fish/aquarium. The mulberry leaves was mixed in 4 formulated diets containing 30% crude protein, and mulberry leaf meal levels were varied; 0, 5, 10 and 15% and fed to Nile tilapia at satiation twice daily for 8 weeks. The results showed that fish fed experimental diets had no significant difference (P>0.05) in specific growth rate, feed conversion ratio, feed efficiency, survival rate, protein efficiency ratio and hepatosomatic index compared with control. Additionally, the mortality of fish challenged by *A. hydrophila* has been recorded for 10 days after challenging. The result showed that fish fed diet containing 15% mulberry leaves had significantly lowest mortality (P<0.05) and had highest relative percentage survival (RPS).

Keywords: Mulberry, Nile tilapia, *Aeromonas hydrophila*, Relative percentage survival

1. Introduction

The Nile tilapia (*Oreochromis niloticus*) was widely cultured in many tropical and subtropical regions of the world, particularly in Asia, Africa and the United States. In Thailand, Nile tilapia was introduced to aquaculture and has become an economically important species for aquaculture due to its fast growing and easily farmed fish. Since tilapia fish farming has been rapidly extended throughout the country, demand of tilapia feed is continuously increased. This has caused a problem of high-priced feed as well as insufficient nutrition [1]. In aquaculture systems the increasing price of feeds is considered one of the most important factors that limit profitability [2]. Fish feed account for at least 60% of the total cost of production [3]. Additionally, Nile tilapia farming also faced of the infectious diseases problems, especially bacterial infection including *Aeromonas hydrophila* which has been considered to be a major problem leading to economic losses in aquaculture. Antibiotics have been used to control diseases in aquaculture for a long time. However, improper use of this kind of substance would appear to have seriously damage to aquatic animals, consumers and environment. Therefore, it is need to find out an alternative resource that is not only effective against fish pathogens but also cheap, safe and readily use as artificial fish feed for aquaculture.

Mulberry (*Morus alba* Linn.) plant is cultivated for sericulture industry and their leaves contain a good quantity protein (21.1%) which can be used as a total substitute (100%) for dietary fish meal [4]. Mulberry leaves are palatable, non-toxic, and nutritious and protein, carbohydrate, calcium, iron, ascorbic acid, carotene, vitamin B-1, folic acid and vitamin D [5]. Mulberry leaf extracts which contain rutin, quercetin, isoquercetin and other flavonoids have been shown to inhibit oxidative modification of LDL and may reduce artherosclerosis [6]. Therefore, the aim of this work was to investigate the effects of mulberry leaves on growth performance, feed conversion efficiency and resistance against *A. hydrophila* in Nile tilapia.

2. Materials and methods

2.1 Antimicrobial activity test of Mulberry

2.1.1 Mulberry extract preparation

Fifty gram of mulberry leaves were dried, ground and immersed in 95% ethanol (1:6 w/v) and kept at room temperature for 7 days. The extract was centrifuged and filtered through Whatman no: 1 filter paper. The filtrate was collected and then evaporated using a rotary vacuum evaporator and stored at 4°C until using.

2.1.2 Bacteria preparation

Aeromonas hydrophila was isolated from hybrid catfish by streak plate technique on Tryptic Soy Agar

(TSA; Himedia). The species of bacteria was identified by morphology using Gram's stain and biochemical properties using API 20E strip (Biomerieux). The bacteria strain was cultured on TSA at 37°C overnight. The isolated colonies were cultivated in Tryptic Soy Broth (TSB; Himedia) and incubated with shaker at 34°C for 18 h. The turbidity of bacteria was adjusted to equivalent to 0.5 McFarland standards (1.5 x 10^8 CFU/ml).

2.1.3 Antimicrobial activity by disc diffusion method

Antibacterial activity of the ~~extracts~~ against *A. hydrophila* was conducted by disc diffusion method [7]. Briefly, the prepared bacteria were inoculated onto Mueller Hinton Agar (MHA; Himedia) surface and distributed evenly with sterile L-shaped glass rod. The sterile paper discs of 5 mm diameter were placed on inoculated agar plate and then 20 µl of herbal extract was impregnated on disc. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone surrounding the discs after incubation at 37°C for 24 h. All tests were carried out in triplicates and expressed in millimeter.

2.1.4 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of crude ~~extracts~~ against *A.hydrophila* were determined by serial two fold dilution method [7]. Briefly, one ml of TSB and one ml of graded doses of crude extract were added to each test tube. After that, one ml of suspended *A.hydrophila* was inoculated to these test tubes followed by incubation at 37°C for 24 h. The final concentration of mulberry extract was 11140, 5170, 2785, 1392.5, 696.25, 348.13, 174.06, 87.03, 43.52 and 21.76 ppm. The tube with the lowest concentration with no visible growth of the bacteria was considered as MIC. Two test tubes including the extract with no bacteria and bacteria with no the extract were used as negative control and positive control, respectively. All tubes with no visible growth were used to examine for presence or absence growth by plating onto agar plate and incubation at 37°C 24 h. The MBC was recorded as the lowest concentration with no detectable growth.

2.2 Experimental diet

Fresh Mulberry leaves were collected from Nong Khai province, Thailand. The leaves were oven-dried at 60°C for 3 days. The dried leaves were grounded into fine powder and analyzed for its proximate analysis using the method of AOAC (1980). Four isonitrogenous diets (30% crude protein) containing 0% (control), 5%, 10% and 15% mulberry leaves were formulated. The composition and proximate analysis (AOAC, 1980) of the diets are shown in Table 1. All ingredients were mixed thoroughly in a Hobart mixer, water was then added, and mixed for 5 min. After that the mash was extruded in a meat grinder with a 3-mm diameter. The moist pellets were oven-dried at 60°C for 12 h, store in plastic bags and kept in freezer until used.

Table 1 Ingredients and proximate composition of experimental diets

Ingredients	Level of Mulberry leaves (%)			
	0	**5**	**10**	**15**
Fish meal (54.08%)	25	25	25	25
Soy bean meal (45%)	29.49	27.41	25.34	23.25
Mulberry leaf (19.38%)	0	5	10	15
Rice bran	20	20	20	20
Corn meal	13	13	13	13
Rice Husk	9.51	6.58	3.66	0.75
Soy bean oil	1	1	1	1
premix	2	2	2	2
Total	100	100	100	100
Proximate composition by analysis (% dry weight on basis)				
Moisture	10.59	10.63	8.71	8.75
Protein	31.01	30.34	31.18	30.94
Fat	7.83	7.99	8.89	8.28
Ash	11.66	11.27	11.20	10.59
Fiber	10.27	9.32	11.10	11.39
Digestible energy (Kcal/100 g)	259.06	262.20	269.03	265.95
Gross energy (Kcal/100 g)	366.52	371.69	378.64	376.17
DE/P (kcal/protein)	8.35	8.64	8.63	8.60

* Gross energy = (%Protein ×5.64)+(%Lipid×9.44)+(%NFE×4.11) (NRC, 1993)

* Digestible energy = (%Protein ×4)+(%Lipid×8.1)+(%NFE×2.5) (NRC, 1993)

2.3 Experimental fish

Nile tilapia (*Oreochromis niloticus*) were obtained from hatchery of fisheries science program, Khon Kaen University, Nong Khai Campus, Nong Khai Province. Fish were acclimatized in the laboratory condition for one week before starting the feeding trial. At the beginning of the experiment, 12 glasses aquaria (0.45 x 0.90 x 0.45 m, water volume 150 liters) were each stock with 20 fish with an average weight of 2.34±0.82 g. and length of 5.56±0.23 cm. Each experimental diet was fed to fish in three aquaria. About 50% of the water in all the experiment aquaria was changed daily. The fish were fed twice daily at 09:00 and 15:00 with apparent satiation with 0% (control), 5%, 10% and 15% mulberry leaves diet for 8 weeks. Satiation was determined from observation of acceptance and refusal of feed.

2.4 Growth performance

At the end of the feeding trial, the fish were weighed and data including weight gain, average daily weight gain (ADG), specific growth rate (SGR), survival rate (SR), feed intake FI), feed conversion ratio (FCR), feed efficiency (FE), protein efficiency ratio (PER) and hepatosomatic index (HSI) were calculated.

2.5 Challenge experiments

After 8 weeks, 10 fish (of each group was randomly collected and challenged by intraperitoneal injection with 0.2 ml of *A. hydrophila* culture containing of 0.3 CFU, which is the lethal dose for the tilapia. Mortality of fish was observed for 10 days post challenge. Relative Percent Survival (RPS) was calculated followed by Ellis and Large [8].

2.6 Statistical analysis

Data from each treatment were subjected to one-way analyses of variance (ANOVA), using SPSS for windows. Means were compared after analysis of variances by Duncan's New Multiple Range Test ($P = 0.05$). The level of significance was chosen at $P \leq 0.05$, and the results are presented as mean ± standard deviation.

3. Results

3.1 Antimicrobial Activity of Mulberry

The result of antibacterial screening performed by disc method was 10.77±0.25 mm in diameter of inhibition zone. The result of minimum inhibitory concentration (MIC) was 2785 ppm. There was no the minimum bactericidal concentrations (MBC) observed in any concentration of mulberry extract. The results of antimicrobial activity assay of mulberry extract against *A. hydrophila* were presented in Table 2.

Table 2 Inhibition zone, MIC and MBC of mulberry against to *Aeromonas hydrophila*

Parameter	Result
Inhibition zone (mm.)	10.77+0.25
MIC (ppm)	2785
MBC (ppm)	-

3.2 Growth performance and Relative percentage survival (RPS)

Over the 8 weeks experiment, all fish adapted well to the experimental system, and no disease or water quality problems were observed during the study. The rates of fish survival ranged from 95.00±5.00% to 81.67±2.33% and showed no significant difference ($P>0.05$) among four dietary groups. The effects of dietary mulberry leaves levels on weight gain, average daily weight gain, specific growth rate, survival rate, feed intake, feed conversion ratio, feed efficiency, protein efficiency ratio and hepatosomatic index of tilapia were not significantly different among the groups ($P>0.05$). After the challenge test, the lowest mortality rate was found in fish fed diet containing 15% mulberry leaves (36.67±5.77) with significant difference among all treatments ($P<0.05$). Fish fed diet containing 15% mulberry leaves also showed the highest relative percentage survival (RPS). All data were presented in Table 3.

Table 3 Weight gain, average daily weight gain, specific growth rate, survival rate, feed intake, feed conversion ratio, feed efficiency, protein efficiency ratio hepatosomatic index and RPS after 8 weeks

Parameters	Level of mulberry leaves (%)				*p*-value
	0	5	10	15	
Weight gain (g)	12.35±0.69	12.73±2.22	12.54±1.50	12.05±0.46	0.941^{ns}
ADG (g/fish/day)	0.22±0.01	0.23±0.04	0.22±0.02	0.22±0.01	0.966^{ns}
SGR (%/day)	3.24±0.08	3.36±0.29	3.27±0.18	3.26±0.04	0.858^{ns}
Survival rate (%)	90.00±8.66	86.67±7.64	95.00±5.00	81.67±2.33	0.235^{ns}
FI (g)	0.45±0.03	0.44±0.04	0.43±0.03	0.49±0.03	0.202^{ns}
FCR	2.12±0.29	2.04±0.49	1.92±0.17	2.36±0.19	0.420^{ns}
FE	0.48±0.06	0.51±0.13	0.52±0.04	0.43±0.03	0.466^{ns}
PER	1.54±0.21	1.68±0.43	1.68±0.15	1.37±0.11	0.455^{ns}
HSI (%)	1.74±0.40	1.63±0.56	1.42±0.32	2.02±0.53	0.182^{ns}
Mortality (%)	73.33±5.77^{a}	56.67±5.77^{b}	53.33±5.77^{b}	36.67±5.77^{c}	0.000*
RPS	-	22.72	27.27	50	-

*Remark: Values receiving different superscript are statistically significant (P<0.05).

4. Discussion

Many herbs extracts were investigated on their antimicrobial activity against some fish pathogenic bacteria for examples, pomegranate peels (*Punica granatum* L. var.), Indian almond leaf (*Terminalia catappa* L.) [9,10], fresh garlic (*Allium sativum* Linn.), Japanese green tea (*Camellia sinensis*) and piper leaf (*Piper sarmentosum*) extracts [10], garlic extract [11] and Sirindhornvallee (*Bauhinia sirindhorniae*) extract [12] showed positive inhibition against *A. hydrophila*. However, there is not much information on the study of antimicrobial activity of mulberry (*Morus alba* Linn.) extract, against fish pathogen. Mulberry, as a non-toxic natural therapeutic plant has been highlighted in various scientific investigations, especially its biological activity and medicinal worth [13]. In this present study, the antibacterial activities of mulberry extracts against *A. hydrophila*, a major fish pathogenic bacteria were applied.

Previously, Chaitali Ravi Niratker et al [14] studied on antimicrobial activity of leaf extract of mulberry (*Morus indica*) against different pathogens. The results showed significant antimicrobial activity against *Staphylococcus aureus*, *Aspergillus niger* and *Penicillium* sp. It has been reported that the antimicrobial activity of ethanol extract of *M. alba* were higher than those of water extract. The MIC and MBC values for the ethanol extracts from leaves against *Bacillus subtilis* was 1.563 mg/ml and 3.125 mg/ml, respectively [15]. *M. alba* also shows good activity against *S. aureus*, *K. pneumoniae* and *E. coli* (10 mm) [16] and also against *A.hydrophila* (10.77 mm) in this study. Additionally, both the crude extract and fraction of *M.alba* inhibited the growth of *S.aureus*. The ethanol crude extract and the ethanol with methanol fraction highly inhibited the growth of *S. aureus* [17].

Additionally, the crude protein content was almost analogous (30%) (Table 1) in the four test feeds (0%, 5%, 10% and 15% mulberry leaves substitution). This was higher than the CP values of 25 % reported by Kamal et al. [18], Based on a review of several studies, recommended 30-35% dietary protein as the optimal level for tilapia. Table 2 shows the growth performance, feed conversion efficiency survival and hepatosomatic index of the tilapia fed varying dietary levels of mulberry leaf for 8 week. The present study indicates that mulberry leaf meal can be incorporated in the diet of tilapia up to a level of 15% without significant negative effect on growth performance and feed utilization. It is clearly shown (Table 3) that all the tested growth parameters (weight gain, ADG, SGR) showed no significant differences between the four test feeds. Kamal et al. [18] reported that ADG, body weight gain and SGR of Nile tilapia was higher when fed with diet replacement of soy bean meal by 50% or 22% while the lowest of those parameters were found in fish fed when replaced with 100% or 44% of mulberry leaf in diet. Bag et al. [19] examined the suitability of mulberry (*Morus alba*) leaf meal as a complete diet for sting fish (*Heteropneustes fossilis*, Bloch). They found that the feed prepared from mulberry leaf was proved to be the better in terms of survival rate, FCR and SGR than other two feed prepared from groundnut oil cake and dried earthworms. The mulberry leaf also enhanced feed efficiency and GSI. Moreover, use of mulberry leaf as a complete diet provided most favorable limnological condition suitable for culture of sting fish and is expected to bring down the cost of fish production. Likewise, the results of the present studies indicated that mulberry leaf can be not only used as an essential ingredient in feed for fish species but also can enhance the immunity power against common fish pathogen.

5. Conclusions

The present study revealed that the antimicrobial activity of mulberry (*Morus alba* Linn.) leaves extract was found to be against *Aeromonas hydrophila*. It may be recommended as a dietary supplement at 15% in order to improve diseases resistance. Further studies on the *in vivo* efficiency of this herb in order to enhance fish immunity and its toxic are needed.

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