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Effects of Dietary Waterlily (*Nymphaea pubescens*) Stamen Extract on Growth Performance and Intestinal Morphology of Common Lowland Frog (*Rana rugulosa*)

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Abstract

The effects of diets containing various levels (0, 1, 3 and 5%) of waterlily (*Nymphaea pubescens*) stamen extract (NPSE) on growth performance and intestinal morphology were investigated in common lowland frog (*Rana rugulosa*) with an initial weight of 16.09±0.50 g. After 11 weeks of feeding, growth parameters were evaluated. The results showed that the frogs fed with diets supplemented with 3 and 5% of NPSE exhibited significantly higher weight gain, specific growth rate and feed conversion ratio than frogs fed diets supplemented with 0 and 1% of NPSE ($P<0.05$). Significant differences were not observed in survival rate, hepatosomatic index and intestinosomatic index among the all diet groups ($P>0.05$). Villi heights, villi widths and the thickness of intestinal muscle layers in posterior intestine were significantly increased in frogs fed the diets containing NPSE ($P<0.05$). Observations on histology of anterior intestine found a significant increase in villi heights and circular muscularis thickness in the treated groups compared to the control group ($P<0.05$). However, villi widths and longitudinal muscularis thickness of anterior intestine did not affect by dietary treatment ($P>0.05$). Feeding behavior and feed acceptability of the experimental groups were the same as the control group. The optimal levels of NPSE observed in this present study were ranged between 3-5%. Our findings suggest that NPSE can be applied in the diets as a growth promoter in common lowland frog.

Keywords : *Common lowland frog, Rana rugulosa, Waterlily Nymphaea pubescens, Growth performance, Intestinal morphology.*

1. Introduction

Thailand is one of the frog producers and exporters in the world. It is estimated that cultured frogs produced by Thai farmers were approximately 1,614 ton in 2012 and those frogs are sold both in the domestic and international markets (1). Three main types of frog products sold in the markets are frog legs, live frogs and educational and scientific purposes (2).

In Thailand, frog cultivation practices can be divided into the following types: pond, ditch and cage (1-3). Intensive frog culture system is generally performed by Thai farmers (2,3). By this practice, frogs are grown in high density and may have many problems with stress, limited space, inadequate diet, cannibalism, predators and water quality (3,4). Thus, good management of frog diet and health should be attained.

Feed additives are commonly used by mixing in animal feeds for the improvement of growth and health status (5-7). The use of synthetic substances could produce harmful effects on animals, consumers as well as the environment (5-7). This knowledge leads researchers to find novel feed additives which are obtained from plants or other natural products (5-7).

Waterlily, a perennial aquatic herb, is widely distributed throughout Southeast Asia including Thailand. All parts of this plant are useful for medicinal purposes (8). Pharmacological investigations revealed that waterlily has antioxidant, anti-inflammatory, anti-diabetic and antibacterial activities (8-12). The main classes of phytochemical compounds found in waterlily are alkaloids, flavonoids, phenolics, tannins, saponins, and steroids (8-10). It has also been shown that quercetin, gallic acid, kaempferol and nuciferine were isolated and identified from waterlily. (13). In aquaculture production, Sivani et al. (14) found that fish diets mixed with *Nymphaea* meal as an alternative protein source can produce better growth rates and survival in common carp (*Cyprinus carpio* L.) when compared to the basal diet. Our recent research indicated diets supplemented with the leaf extract of waterlily significantly enhanced growth performance and feed efficiency of Nile tilapia without unwanted side-effects during the experimental period (15). As many researchers have been reported on growth promoting potential of waterlily in some species of fish (14,15), however, its use in frog production as a feed additive is scarce. The aims of this research were conducted to study the effects of diets containing various levels (0, 1, 3 and 5%) of waterlily stamen extract (NPSE) on growth performance and intestinal morphology of common lowland frog (*Rana rugulosa*).

2. Materials and Methods

2.1 Plant collection and extraction

Flowers of waterlily used in this experiment were obtained from Kantharalak District, Srisaket Province, Thailand. Waterlily stamens were collected, cleaned and dried in hot air oven at 50^{o}C. Dried stamens were extracted using 70% ethanol for 14 days. The extract was then filtered using Whatman paper No 1., removed the solvent by rotary evaporator which was maintained at 50^{o}C and 90 rpm. The extract was dried by lyophilizer and kept in refrigerator until used.

2.2 Frog rearing conditions

Common lowland frogs with an initial weight of 16.09±0.50 g were obtained from Ubon Ratchathani Fisheries

Cooperatives, Ubon Ratchathani, Thailand. They were distributed in earthen ponds ($0.5 \times 1.5 \times 0.5$ m^3) in a completely randomized design using 4 treatments and 3 replications. Each replication consisted of 30 frogs.

The basal diets were obtained from the commercial feed company containing not less than 30% crude protein and 4% lipid. Each level (1, 3 and 5%) of NPSE was added to the basal diets and mixed together by using egg white as a binder (16). Proximate composition of dietary were analyzed for their nutrient ingredients including moisture (%), ash (%), crude protein (%CP) and crude lipid (%CL) by the procedures described by the Association of Official Analytical Chemists (AOAC International 2012) and the data of proximate analysis are summarized in Table 1. Frogs in each treatment were fed the diets containing NPSE for 11 weeks with a rearing rate of 3.5% of live weight.

Table 1. Proximate composition of the experimental diets.

Proximate composition (%)	**Diets**			
	Control	**1%NPSE**	**3%NPSE**	**5%NPSE**
Moisture (%)	4.14	5.12	6.11	6.09
Ash (%)	13.90	13.72	14.11	14.00
Crude protein (%CP)	31.48	31.69	32.41	32.37
Crude lipid (%CL)	6.94	6.97	7.11	7.09

Remarks: Control = the basal diet, NPSE = *Nymphaea pubescens* stamen extract.

2.3 Effects on growth parameters

At the end of the treatment period, frogs were fasted for 24 hr. Growth parameters were evaluated by using following equations:

Weight gain (WG) = final frog weight (g) – initial frog weight (g).

Average daily growth (ADG, g/d) = (final wet weight- initial wet weight)/ experimental days.

Specific growth rate (SGR, % d^{-1}) = 100× [In final wet weight (g) – In initial wet weight (g)]/experimental days.

Feed conversion ratio (FCR) = feed intake (g)/ weight gain (g).

Survival rate (SR, %) = 100× (final number of frog/initial number of frog).

Ten frogs from each replication were randomly collected to dissect internal organs including liver and intestine and calculate the relative organ weights by using following equation.

Relative organ weight (%) = 100× (weight of organ (g)/weight of fish (g)).

Ponds were cleaned every 5 days. Dissolved oxygen, pH and water temperature were maintained in standard conditions (4). Dead frogs, if present, were removed immediately and noted.

2.4 Effect on intestinal morphology

At the end of experiment period, frogs were fasted for 24 h and weighted. Double-pitched frogs were cut the abdominal wall carefully to obtain the gastrointestinal tracts using sharp scissors. The intestines were collected, cleared from adipose tissues and subsequently divided into two parts: anterior intestine and posterior intestine. The specimens were fixed in 10% neutral buffered formalin, dehydrated in ethanol, embedded in paraffin and cut into 5 μm thick. The sections were then strained with haematoxylin and eosin (H&E). The thickness of intestinal wall, the height of villi and the thickness of villi were measured from the intestine slides under the light microscope connected to the computer running DinoCapture 2.0 software and recorded.

2.5 Statistical analysis

Data are expressed as means ± standard error of mean (SEM). Parameters of frog growth were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. Significant differences were set at $P<0.05$.

3. Results

3.1 Effects on growth parameters

During the 11 weeks of experiment, growth parameters of reared frogs were evaluated. The results showed that the frogs fed with diets supplemented with 3 and 5% of NPSE exhibited significantly higher WG, ADG and SGR than frogs fed diets supplemented with 0 and 1% of NPSE ($P<0.05$). FCR values diets were significantly decreased in frogs fed with 3 and 5% NPSE compared to the control ($P<0.05$). Significant differences were not observed in the survival rate, hepatosomatic index as well as intestinosomatic index among the all diet groups ($P>0.05$). In addition, feeding behavior and feed acceptability of the experimental groups were the same as the control group. The suitable levels of NPSE observed in this research were ranged between 3-5%. The effects of dietary supplementation with NPSE on growth of frogs are summarized in Table 2 and the average body weights of frogs in each treatment weighed in every other week are shown in Figure 1.

Table 2. Effects of dietary supplementation with NPSE on growth performance of common lowland frogs.

Parameters	Diets			
	Control	1% NPSE	3%NPSE	5%NPSE
Initial weight (g)	16.10±0.57	16.09±0.70	16.10±0.42	16.10±0.32
Final weight (g)	58.40±0.92^{a}	70.80±2.63^{b}	76.60±1.36^{c}	81.40±5.07^{c}
WG (g)	41.32±0.74^{a}	54.64±1.67^{b}	60.52±0.86^{c}	65.32±2.69^{c}
ADG (g/d)	1.37±0.03^{a}	1.80±0.04^{b}	2.03±0.06^{c}	2.20±0.06^{c}
SGR (% d^{-1})	4.18±0.04^{a}	5.34±0.07^{b}	5.40±0.06^{b}	5.48±0.05^{b}
FCR	2.39±0.06^{a}	1.85±0.12^{b}	1.63±0.14^{b}	1.52±0.12^{c}
SR (%)	99.00±1.00	99.00±1.00	97.00±3.00	100.00±0.00
HSI (%)	2.40±0.19	2.51±0.14	2.47±0.49	2.84±0.12
ISI (%)	8.54±1.15	8.81±1.65	8.16±1.26	8.25±1.19

Remarks: Data were presented as mean±SEM. Values with different superscripts ($^{a\text{-}c}$) within the same row are significantly different (P<0.05). NPSE = *N. pubescens* stamen extract; WG = weight gain; ADG = average daily growth; SGR = specific growth rate; SR = survival rate; HSI = hepatosomatic index; ISI = intestinosomatic index.

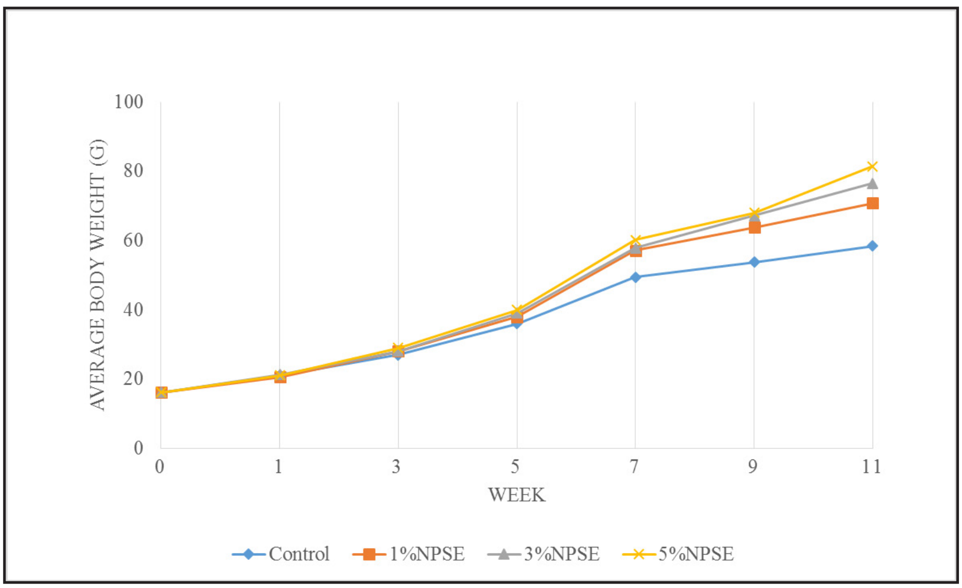

Figure 1. Average body weights of frogs fed the basal diet (control) and the diets containing 1%, 3% and 5%NPSE for 11 weeks. NPSE = *N. pubescens* stamen extract.

3.2 Effect on intestinal morphology

Observations on intestinal morphology of frog fed dietary NPSE found a marked increase in villus height, villus thickness as well as the thicknesses of longitudinal muscularis and circular muscularis of posterior intestine compared with the control (P<0.05). In anterior intestine, increased villi heights and circular muscularis thickness were found in the experimental groups (P<0.05). However, there were no significant differences in villi widths and longitudinal muscularis thickness in anterior intestine of frogs fed the diets containing NPSE (P>0.05). The effects of the diets mixed with NPSE on intestinal morphology of frogs are summarized in Table 2 and the cross sections of anterior and posterior intestines obtained from frogs fed the experimental diets are shown in Figures 2 and 3.

Table 3. Intestinal morphology of frogs fed the diets containing NPSE for 11 weeks.

Diets	Anterior intestine				Posterior intestine			
	Villus height (µm)	Villus thickness (µm)	Longitudinal muscularis thickness (µm)	Circular muscularis thickness (µm)	Villus height (µm)	Villus thickness (µm)	Longitudinal muscularis thickness (µm)	Circular muscularis thickness (µm)
Control	2843.44±145.04^{a}	1073.40±65.78	234.85±20.99	686.30±50.22^{a}	2442.07±98.13^{a}	734.66±35.04^{a}	214.64±10.50^{a}	660.64±52.70^{a}
1% NPSE	4150.14±202.50^{b}	907.33±81.53	314.27±69.47	869.09±107.90^{ab}	3395.02±307.68^{b}	1033.66±101.16^{b}	254.97±6.91^{b}	989.96±55.65^{c}
3% NPSE	3283.91±434.48^{ab}	1047.44±75.19	270.22±13.41	942.16±54.33^{b}	2461.88±244.17^{a}	741.13±46.51^{a}	270.39±16.95^{b}	714.86±77.07^{ab}
5% NPSE	3194.94±392.14^{ab}	974.04±63.36	270.84±14.26	1004.99±60.76^{b}	3294.11±261.25^{b}	941.19±70.59^{b}	259.35±12.15^{b}	874.61±60.50^{bc}

Remarks : Data were presented as mean±SEM. Values with different superscripts ($^{a\text{-}c}$) within the same column are significantly different (P<0.05). NPSE = *N. pubescens* stamen extract.

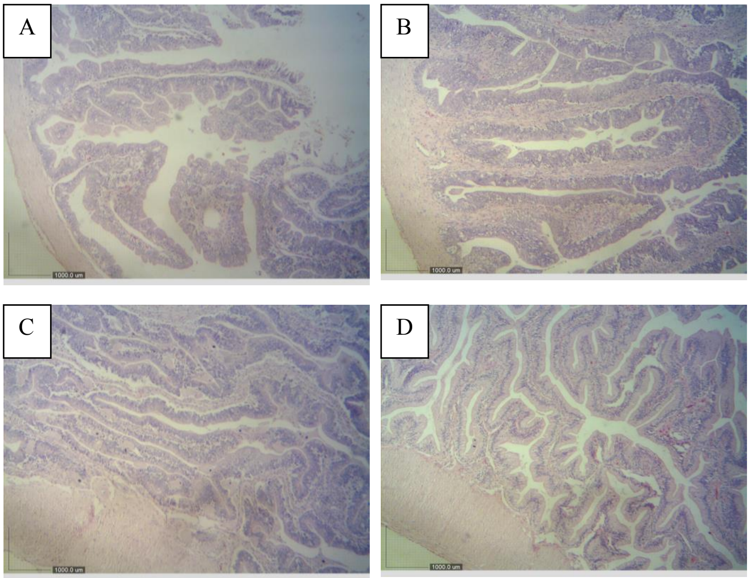

Figure 2. Portions of anterior intestine of frogs fed the diets containing NPSE for 11 weeks. NPSE = *N. pubescens* stamen extract. A = section of anterior intestine collected from frog fed the diets incorporated with the control diet, B = section of anterior intestine collected from frog fed the diets incorporated with 1% NPSE, C = section of anterior intestine collected from frog fed the diets incorporated with 3% NPSE, D = section of anterior intestine collected from frog fed the diets incorporated with 5% NPSE. Scale bar = 1000 µm.

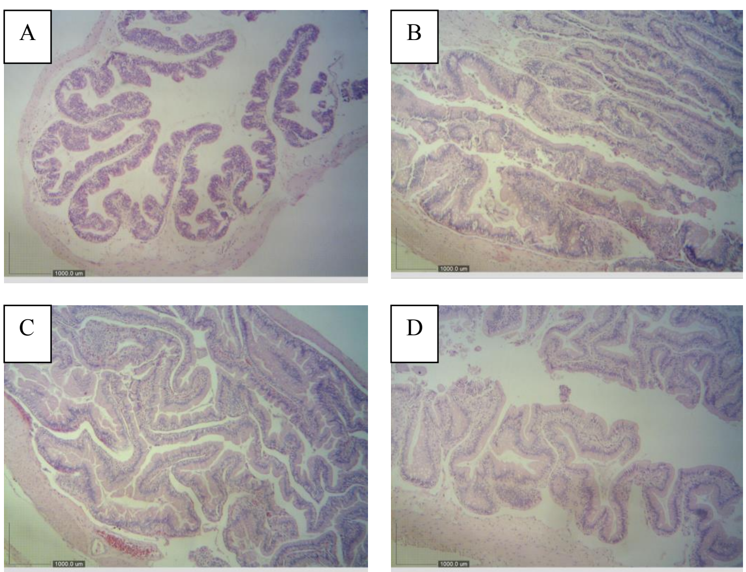

Figure 3. Portions of posterior intestine of frogs fed the diets containing NPSE for 11 weeks. NPSE = *N. pubescens* stamen extract. A = section of posterior intestine collected from frog fed the diets incorporated with the control diet, B = section of posterior intestine collected from frog fed the diets incorporated with 1% NPSE, C = section of posterior intestine collected from frog fed the diets incorporated with 3% NPSE, D = section of posterior intestine collected from frog fed the diets incorporated with 5% NPSE. Scale bar = 1000 µm

4. Discussion

4.1 Effects on growth parameters

Medicinal plants and their derivatives are generally used to alleviate various diseases in human. In addition, the application of many herbal plants to animal feeds for improving growth and health of cultured animals is rapidly increased in the last few decades (5-7). It is widely accepted that the utilization of natural compounds and metabolites is safer than the synthetics (5-7). Thus, our research was aimed to study the effects of NPSE on growth performance of common lowland frogs. We found that frogs fed the diets containing NPSE significantly increased final weight, WG, ADG and SGR. In addition, FCR values of the experimental frogs were lower than those of the control frogs, indicating that NPSE could be used as feed additive in aquatic feeds to promote productivity. Our study also showed that 3-5% of NPSE were suitable for use in the frog diets.

Few data are focused on the use of herbal plants in frog cultivation. Choshasee et al. (17) found that diets supplemented with *Centella asiatica* and *Zingiber montanum* at the concentrations of 0.5, 1.5 and 2.5% did not produce any change in growth and health of common lowland frog when compared to the control diet. However, frog diets mixed with 2.5% of *C. asiatica* and 1.5 and 2.5% of *Z. montanum* effectively inhibited the growth of *Flovobacterium multivorum* in subcutaneous tissue of treated frogs. In addition, Klahan and Sirithanawong (18) reported that frogs and tadpoles fed the diets supplemented with bromelain enzyme extracted from crown of pineapple at the levels of 0.25, 0.5 and 1 ml/g feed significantly increased protein digestibility in a dose dependent manner. The optimum levels of bromelain observed were 0.5 and 1 mg/g feed (18). Dietary supplementation with dried powers of *Pueraria mirifica* tubers (20 g/kg feed) and *Butea superba* roots (20 g/kg feed) significantly enhanced the growth of frog tadpoles and improved the developmental change from tadpole to frog (19). Suriya et al. (20) observed that diets incorporated with the extracts of *Spirulina platensis* and *Phyllanthus emblica* significantly increased the survival rate of tadpole when compared with the control diet.

There is evidence that growth promoting properties of medicinal plants could be attributed to the enhancement of palatability of feeds, feed acceptability, nutrient digestion and absorption as well as the functions of digestive enzymes (amylase, protease and lipase), resulting in the increases of appetite, food consumption and growth (5-7). Immunomodulatory, antifungal, antibacterial, antiviral and antioxidant properties of herbs could also maintain and support health and performance of animals, due to the prevention of various opportunistic diseases (5-7).

Similar to the actions of herbal plants described above, the mechanisms of growth promoting effects of NPSE on frog observed might be attributed to the improvement of feed intake, palatability and digestive enzyme functions, leading to better growth performance and feed utilization efficiency (5-7).

Several classes of plant secondary metabolites such as alkaloids, flavonoids, saponins, triterpenoids, steroids and essential oils have been isolated, identified and tested for their growth promoting properties in certain species of aquatic animals (5-7). Previous investigations indicated that alkaloids, flavonoids, phenolics, saponins, and steroids are the main compounds found in waterlily. (8-10). Thus, growth promotion of waterlily observed in this present investigation could be attributed to the actions of alkaloids, flavonoids, phenolics or saponins. Frog fed the diets containing 3-5% NPSE resulted higher growth than those fed on the diet containing 1% NNSE. This result may be due to the amount of phytochemicals contained in 3-5% NPSE could reach the optimal levels for supporting growth and general well-being of frog.

It has been reported that plants used in animal feed production may contain some anti-nutritional components such as tannin which would decrease growth and feed utilization efficiency of animals. Becker and Makkar (21) revealed that common carp fed the diet mixed with 2% quebracho tannin for 84 days did not exhibit any side effects. On the other hand, on day 28 of the experimental period, carp fed the diet containing 2% tannic acid reduced feed intake, metabolic growth rate and oxygen consumption compared to control (21). Thus, further studies are required to isolate, identify and test some active compounds or anti-nutritional contents found in waterlily for testing their biological effects such as growth improvement and immunostimulation in aquatic animals.

4.2 Effect on intestinal morphology

To the best of our knowledge, this present study is the first to demonstrate the effects of the diets containing NPSE on frog intestinal morphology. We found that dietary NPSE potentially enhanced the thicknesses of longitudinal muscularis and circular muscularis, villi heights as well as villi widths in anterior and posterior portions of frog intestines.

It is generally accepted that the study of intestinal morphology could be useful for understanding the effects of medicinal plants and their compounds on digestion and absorption of nutrients (22-24). The increases of intestinal villi lengths and widths could enhance the absorptive surface area of the gastrointestinal tract and these assessments are positively associated with feed uptake of aquatic animals (22-24). Additionally, increased thickness of the intestinal muscle layers would also support the movement of digestive tract to evoke nutrient digestibility and moisture absorbability (24-27). Our data revealed that frogs fed diets supplemented with NPSE increased the height of villi, width of villi and thickness of intestinal muscles when compared with the control. Thus, our findings indicated that NPSE could be used to improve frog growth, which is due to the enhancement of gut absorption area produced by NPSE (24-27).

The mechanisms of action by which natural products stimulate intestinal muscle development and increase intestinal villi lengths and widths in frog intestines are not clear yet. However, several studies hypothesized that medicinal plants and their active ingredients might improve intestinal cell proliferation and differentiation (22-27). Moreover, herbs may also increase intestinal cell division and decrease apoptosis, leading to an increase in thicknesses of intestinal villi and intestinal wall (22-27). Based on these scientific evidences, it is possible to speculate that NPSE could promote cell proliferation and cell division in frog intestines in order to enhance the digestive ability and intestinal morphology in this research (22-27). Thus, improved intestinal morphology would relate to the improvement of growth rate and feed utilization efficiency.

5. Conclusion

Our present study is the first report to indicate that NPSE could be submitted to a novel natural feed additive in frog culture as it can improve growth performance, feed efficiency and intestinal histology of common lowland frog. The optimal concentrations of NPSE observed in this research were ranged between 3-5%.

6. Acknowledgement

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