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Growth Performance and Intestinal Morphology of Common Lowland Frog (*Rana rugulosa*) Fed Diets Supplemented with Lotus (*Nelumbo nucifera* Gaertn.) Stamen Extract

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Abstract

The aims of this research were to study the effect of *Nelumbo nucifera* Gaertn. stamen extract (NNSE) as a natural feed additive on growth performance and intestinal morphology of common lowland frog (*Rana rugulosa*). Frogs with an average of 16.35 ± 0.68 g were fed with diets containing 0, 1, 3 and 5% of NNSE for 11 weeks. The results showed that final weight, weight gain, average daily gain and specific growth rate of frogs fed the diets incorporated with NNSE were significantly higher than those of frogs fed the basal diet ($P < 0.05$). In addition, frogs fed diets mixed with NNSE significantly decreased feed conversion ratio compared to the control ($P < 0.05$). No significant changes occurred in the survival rate, hepatosomatic index and intestinosomatic index among the groups ($P > 0.05$). Villi heights, villi widths, the thicknesses of villi, longitudinal muscularis and circular muscularis in the intestines of frogs fed the diets containing NNSE had significantly higher than frogs fed the control diet ($P < 0.05$). Feeding behavior, feed acceptability and health of frogs in the experimental groups were similar to the control group, indicating that NNSE did not have any toxic effect on frog. The optimal concentration of NNSE observed in this investigation was 5%. Thus, our research supports the use of NNSE as natural feed additive in the diet to improve growth and intestinal morphology of frog.

Keywords : *Common lowland frog, Rana rugulosa, Lotus Nelumbo nucifera Gaertn., Growth performance, Intestinal morphology.*

1. Introduction

Many species of frogs have long been consumed by local people in Thailand. Tadpoles, young and adult frogs are generally harvested from agricultural areas for sale in the local markets, resulting in a marked decrease in a number of frog populations in nature (1,2). In addition, several activities of human also affect the extinction of some species of frog including pollution, over-collection for food and habitat destruction (2). Thus, frog cultures have been developed for the production of edible frogs to support the higher demand for human consumption (3-5).

Intensive frog culture practices have major problems with high frog densities, water quality and inadequate nutrition. (2). Under crowded conditions, cultured frogs may also face with a high prevalence of gram negative bacterial infection such as *Aeromonas hydrophila*, *Klebsiella pneumonia* and *Proteus mirabilis*, leading to a development of fatal dermatosepticemia (6,7). Antibiotics are commonly used to treat and prevent several diseases in aquaculture including in frog farming (8). Recent research indicated that subtherapeutic use of antibacterial agents in animal diets may be useful to improve the growth performance of animals (9). However, antibiotic-resistant infections both in humans and animals may be developed (10,11). It is well established that drug- residues could be harmful to people who consumed meat products from drug-treated animals (10,11).

Medicinal plants and their phytochemical contents are economically important as novel materials to synthesis the synthetic drugs, food additives, pesticides, flavor and fragrances (12,13).

Drugs used for the treatment of emerging infectious diseases around the globe are also derived from herbs (12,13). In aquaculture production sections, herbal plants are widely used as growth promoters, appetizers, aphrodisiacs and disease preventers, due to the influence of secondary metabolites including alkaloids, saponins, triterpenoids, flavonoids and essential oils (10,11,14). *Quillaja* saponins, quercetin as well as essential oils have been reported to have growth promoting effects on several cultured fish species by improving growth performance and feed utilization (10,11,14-16).

As mentioned above, the improvement of fish growth and health has been successfully carried out by using herbal plant extracts or isolated plant compounds mixed with the basal diet as natural feed additives. However, the potential utilization of medicinal plants in frog rearing is still limited (1,8,17). Our previous reports indicated that *N. nucifera* Gaertn. leaf and peduncle extracts have a growth promoting effect on fish (18,19). Several active ingredients found in lotus are saponins, phenolics, alkaloids, essential oils and flavonoids (20-24). Therefore, the aims of this research were to study the effects of *N. nucifera* stamen extract (NNSE) as a natural feed additive on growth and intestinal morphology of common lowland frog (*Rana rugulosa*).

2. Materials and Methods

2.1 Plant preparation and extraction

The samples of lotus flowers were collected from the local lotus garden in Don Mod Daeng District, Ubon Ratchathani during rainy season. Lotus stamens were manually collected, cleaned by using tab

water, dried by using hot air oven at 40°C for 24 h and blended to a fine powder by using a home electric blender. Powdered stamens (500 g) were macerated with 70% ethanol (2000 mL) for 7 days at room temperature. The stamen extract was filtered using Whatman paper No. 1 and dried by rotary evaporator which was maintained at 50°C and 90 rpm. The yield of the extract was 16.53% based on dried stamen weight. The extract was kept at 4°C in a refrigerator until used.

2.2 Diet preparation

A commercially-produced pellet containing not less than 30% protein and 4% lipid was used as a basal diet and mixed

with different levels (0, 1, 3 and 5%) of NNSE by using egg white as a binder (25). Then, the samples of the diets were dried in hot air oven at 60°C for 1 day. The diets were kept in the zip-lock bag to prevent humidity and stored in a cool, dry place. The basal and experimental diets were analyzed for nutrient compositions based on dry matter including moisture (%), ash (%), crude protein (%CP) and crude lipid (%CL) by the methods described by the Association of Official Analytical Chemists (AOAC International 2012). The results of proximate composition of the diets are summarized in Table 1.

Table 1. Proximate composition of the experimental diets.

Proximate composition (%)	Diets			
	Control	1%NNSE	3%NNSE	5%NNSE
Moisture (%)	4.04	3.06	3.93	4.86
Ash (%)	13.80	14.33	14.50	13.66
Crude protein (%CP)	31.52	32.92	32.95	31.90
Crude lipid (%CL)	6.94	7.35	7.37	7.33

Remarks: Control = basal diet, NNSE = *N. nucifera* stamen extract.

2.3 Frog preparation

Frogs with an average of 16.35±0.68 g were obtained from Ubon Ratchathani Fishery Cooperatives, Ubon Ratchathani, Thailand and acclimatized to the experimental condition for 1 week. The CRD experiments were divided into 4 treatments with 3 replications (30 frogs per replication). Treatment 1 was fed a basal diet without NNSE as the control group, while treatment 2-4 were fed NNSE as levels of 1, 3 and 5% of diets, respectively. Frogs were reared in the earthen ponds (0.5×1.5×0.5 m³) and fed the experimental diets for 11 weeks on Styrofoam. The qualities of water were maintained in the optimal conditions (temperature,

29.00±2.00°C, pH, 7.20±0.50 and dissolved oxygen 7.00±0.05 mg/L). All ponds were cleaned once every 5 days. Frogs were fed *ad libitum* two times a day and weighted every one week. Dead frogs were noted and removed.

2.4 Effect on growth performance

At the end of the treatment period, frogs were fasted for 24 hr before study. Growth parameters were evaluated using the following formulae.

Weight gain (WG, g) = 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) = $100 \times [\ln \text{ final wet weight (g)} - \ln \text{ initial wet weight (g)}] / \text{experimental days}$.

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

Survival rate (SR, %) = $100 \times (\text{final number of frog} / \text{initial number of frog})$.

In addition, five frogs from each replication were collected to study the relative organ weights of liver (hepatosomatic index, HSI) and intestine (intestinosomatic index, ISI) by using the following equation.

Relative organ weight (%) = $100 \times (\text{weight of organ (g)} / \text{weight of fish (g)})$.

2.5 Effect on intestinal morphology

At the end of feeding trial, frogs were fasted for 24 h and then weighed. The abdominal wall of double-pithed frogs was cut towards to the sternum. The gastrointestinal tract was removed and the samples of intestine were cleared from other organs and weighted. Anterior and posterior parts of the intestines were dissected and fixed in 10% neutral buffered formalin for 24 h, dehydrated in graded ethanol series and embedded in paraffin wax. The tissue samples were cut into 5 µm thick and then stained with haematoxylin and eosin (H&E) for the histological analysis.

To examine the effects of NNSE on intestinal morphology of frogs, villi heights, villi widths, longitudinal muscularis and circular muscularis were evaluated under microscope from each intestine slide using DinoCapture 2.0 software.

2.6 Observations on external appearance and feed acceptability

During the experimental period, external appearance, feeding behavior, feed acceptability and general health of frogs in all groups were observed daily.

2.7 Statistical analysis

Data were analyzed using one-way ANOVA and expressed as mean±standard error of the mean (SEM). The significant differences among the treatments were evaluated using Duncan's multiple comparisons, at the 5% level of significance ($P < 0.05$).

3. Results

3.1 Effect of growth

Growth parameters of frogs fed the diets containing NNSE compared to the control diet are summarized in Table 2. We found that final weight, WG, ADG and SGR of frogs fed the diets incorporated with NNSE were significantly higher than those of frogs fed the control diet ($P < 0.05$). In addition, frogs fed diets mixed with the plant extract at different levels significantly decreased FCR compared with the control ($P < 0.05$). No significant changes occurred in the survival rate, HSI and ISI among the groups ($P > 0.05$). Average body weights of frogs fed the diets supplemented with various levels of NNSE for 11 weeks are shown in Figure 1. We observed that the suitable level of NNSE for use in the frog diet was 5%.

Table 2. Growth performance and survival rate of common lowland frogs fed diets incorporated with NNSE for 11 weeks.

Items	Diets			
	Control	1%NNSE	3%NNSE	5%NNSE
Initial weight (g)	16.60±0.60	16.20±0.86	16.00±0.63	16.60±0.60
Final weight (g)	71.80±4.15 ^a	92.20±4.99 ^b	94.00±1.46 ^b	100.60±1.90 ^c
WG (g)	54.34±0.14 ^a	75.71±0.22 ^b	78.58±0.32 ^b	84.25±0.13 ^c
ADG (g/d)	1.84±0.02 ^a	2.53±0.01 ^b	2.60±0.04 ^b	2.78±0.05 ^c
SGR (%d ⁻¹)	4.96±0.02 ^a	5.78±0.05 ^b	5.90±0.08 ^b	6.10±0.04 ^c
FCR	1.93±0.12 ^a	1.48±0.08 ^b	1.46±0.13 ^b	1.39±0.24 ^c
SR (%)	94.25±0.19	90.75±0.33	91.75±0.65	95.00±0.16
HSI (%)	2.83±0.21	3.08±0.10	3.18±0.39	3.21±0.31
ISI (%)	8.71±1.20	8.94±0.70	9.78±1.10	9.97±1.30

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

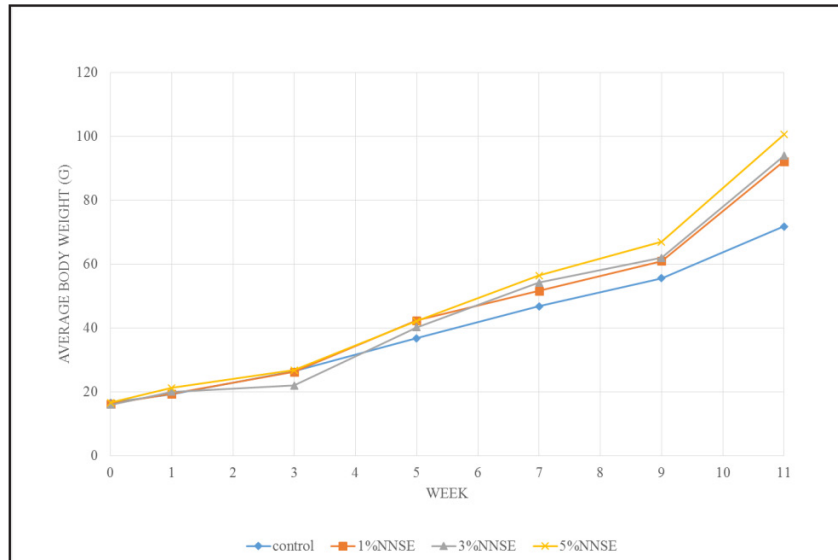


Figure 1. Average body weights of frogs fed the basal diet (control) and the diets containing 1%, 3% and 5%NNSE for 11 weeks. NNSE = *N. nucifera* stamen extract.

3.2 Effect on intestinal morphology

Effects of dietary NNSE on intestinal morphology of frog are summarized in Table 3. Villi heights and

villi widths observed in anterior and posterior intestines of frogs fed dietary supplementation with NNSE were significantly higher than frog fed the diet

without the plant extract ($P < 0.05$). Additionally, the thicknesses of longitudinal muscularis and circular muscularis

significantly increased in NNSE-treated group when compared to control group ($P < 0.05$) (Figures 2 and 3).

Table 3. Intestinal morphology of frogs fed the diets containing NNSE 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 \pm 145.04 ^a	1073.39 \pm 65.78 ^a	234.85 \pm 20.99 ^a	686.30 \pm 50.22 ^a	2442.07 \pm 98.13 ^a	734.66 \pm 35.04 ^a	214.64 \pm 10.50 ^a	660.64 \pm 52.70 ^a
1% NNSE	4988.16 \pm 438.13 ^b	1372.00 \pm 91.77 ^b	300.56 \pm 23.89 ^b	959.55 \pm 84.06 ^b	2715.33 \pm 151.32 ^{ab}	771.94 \pm 44.18 ^a	244.32 \pm 9.50 ^b	739.66 \pm 53.73 ^{ab}
3% NNSE	3616.11 \pm 296.70 ^b	1121.36 \pm 81.29 ^{ab}	272.28 \pm 14.19 ^{ab}	969.02 \pm 116.51 ^b	2762.74 \pm 281.41 ^{ab}	1060.51 \pm 114.02 ^b	220.90 \pm 5.13 ^{ab}	738.09 \pm 53.19 ^{ab}
5% NNSE	3745.34 \pm 358.42 ^b	935.44 \pm 93.77 ^a	232.26 \pm 13.78 ^a	974.67 \pm 69.33 ^b	3064.43 \pm 136.09 ^b	985.37 \pm 63.34 ^b	238.52 \pm 5.59 ^{ab}	915.07 \pm 117.42 ^b

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

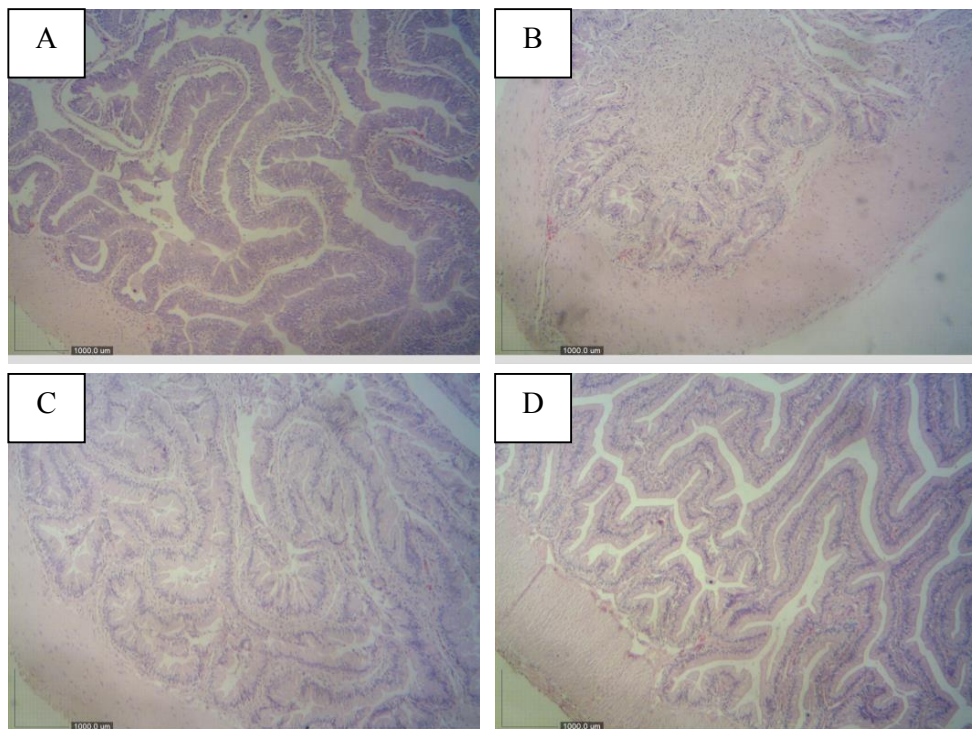


Figure 2. Sections of anterior intestine obtained from frogs fed the diets containing NNSE for 11 weeks. NNSE = *N. nucifera* stamen extract. A = anterior intestine collected from frog fed the basal diet, B = anterior intestine collected from frog fed the diet supplemented with 1% NNSE, C = anterior intestine collected from frog fed the diet supplemented with 3% NNSE, D = anterior intestine collected from frog fed the diet supplemented with 5% NNSE. Scale bar = 1000 μm .

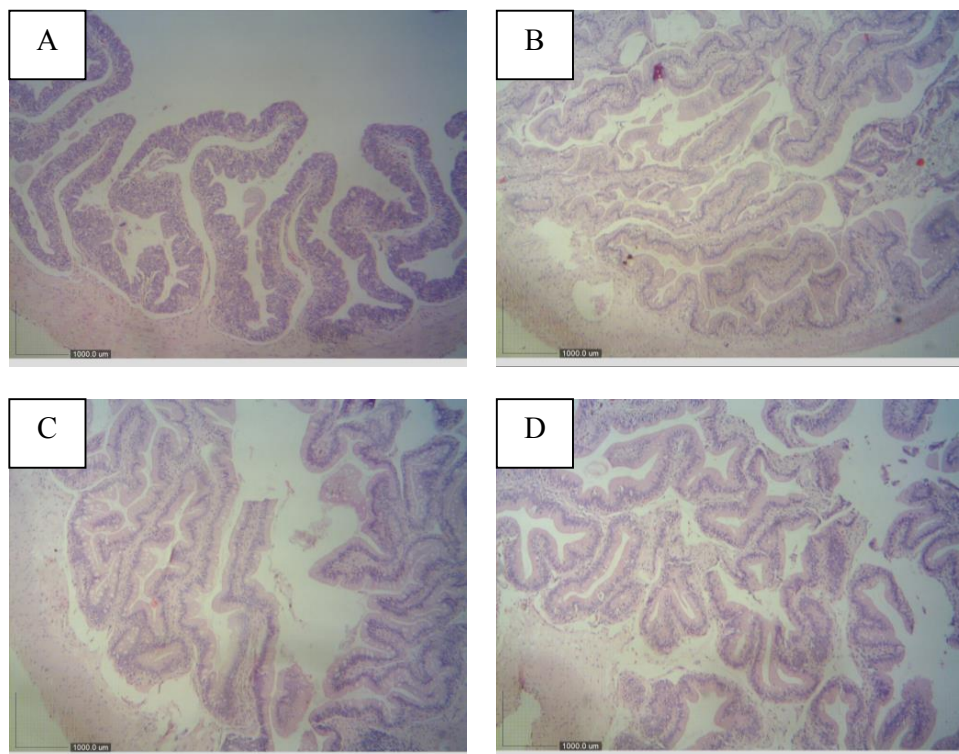


Figure 3. Sections of posterior part of intestines obtained from frogs fed the diets containing NNSE for 11 weeks. NNSE = *N. nucifera* stamen extract. A = posterior intestine collected from frog fed the basal diet, B = posterior intestine collected from frog fed the diet supplemented with 1% NNSE, C = posterior intestine collected from frog fed the diet supplemented with 3% NNSE, D = posterior intestine collected from frog fed the diet supplemented with 5% NNSE. Scale bar = 1000 µm.

3.3 Observations on external appearance and feed acceptability

External appearance, feeding behavior, feed acceptability and general health of frogs in the experimental groups were similar to the control group.

4. Discussion

4.1 Effect on growth

The use of medicinal plants in aquaculture production is markedly increased (8,10,11,14-19). Aquatic feeds supplemented with herbal plants are useful for the improvement of growth performance

and overall health status of various species of aquatic animals (8,10,11,14-19). Our earlier research suggested that leaf and peduncle extracts of *N. nucifera* had growth-promoting effects on fish (18,19). However, the effect of medicinal plants such as *N. nucifera* on growth performance of frogs is still limited and questioned. The aim of this study was to examine the growth-promoting effects of NNSE on some growth indices of common lowland frog. Our findings demonstrated that frogs fed the diet containing NNSE for 11 weeks significantly increased final body weight,

WG as well as SGR compared to the control. In addition, we did not find any signs of toxicity or unwanted side effects in the experimental groups. No significant differences were observed in SR, HSI and ISI. The optimal level of NNSE observed in this research was 5%. Our results suggest that NNSE could be useful for cultured frog as a natural growth promoting agent.

Several research have succeeded in the use of natural feed additives to promote the growth of frogs. Kaewtapee et al. (17) investigated the effects of diets incorporated with dried powers of *Pueraria mirifica* tuber (20 g/kg) and *Butea superba* root (20 g/kg) on frog tadpole growth and development and found that diets containing the dried powders of *P. mirifica* and *B. superba* significantly enhanced body length and body weight of tadpole during 35 days of treatment. In addition, percentages of the developmental change from tadpole to frog at day 35 were significantly increased in the experimental groups, compared to the control group. Boontha et al. (1) studied the effects of dietary supplementation with 5% of *Spirulina platensis*, 5% of *Cladophora* sp. and 5% of *Allium sativum* on growth, gonadosomatic index and phagocytic activity of common lowland frog and they found that the experimental diets did not significantly affect the growth rate of frog when compared to the control diet. However, phagocytic activity of frogs fed the diet mixed with *S. platensis* and *Cladophora* sp. was significantly higher than those in *A. sativum* treated group and basal diet group. Furthermore, frogs fed dietary supplementation with *A. sativum* significantly increased gonadosomatic index when compared to other groups. Kotsuntea et al. (8) reported that growth

parameters and survival rate of tadpole fed the diets containing herbal plant extracts including bitter leaves, guava leaves, basil leaves and star gooseberry combined with bio-extract were significantly greater than those of the diet without the plant extract and bio-extract.

The growth-promoting properties of NNSE mixed with the basal diet in frog observed in this research could be attributed to the improvement of palatability and the increase of nutrient digestion and absorption in the gastrointestinal tract which may partially activate specific digestive enzymes, leading to the enhancement of the growth of frog (10,11,14-16).

Phytochemical contents found in various species of medicinal plants are identified and isolated to test both for their biological and pharmacological activities (10,11,14-16). Several scientific data exhibited that alkaloids, flavonoids, triterpenoids, essential oils and saponins can be used as feed additives for improving growth and feed utilization efficiency of various species of aquatic animals (10,11, 14-16). Previous reports revealed that *N. nucifera* contains alkaloids, flavonoids, triterpenoids, tannins and saponins (20-23). These data led us to hypothesize that alkaloids, flavonoids and saponins found in NNSE could be produced the growth promoting effects on frog in this present investigation (18,19). Anti-viral (20), antioxidative (21), anticancer (22) and anti-inflammatory (23) activities of *N. nucifera* have been examined. The extracts of rhizomes and seeds of this plant were also tested for their immunomodulatory potential (24). These biological effects of *N. nucifera* could protect frogs from numerous infectious diseases and support

the optimal growth of frog. It was found that 2% quebracho tannin, a condensed tannin mixed diets did not produce any side effects on common carp (26). However, carp fed the diets incorporated with 2% tannic acid, hydrolysable tannin, decreased average metabolic growth rate, feed intake and oxygen consumption after day 28 of the experimental period (26). Thus, nutritionists should be concerned about some anti-nutritional ingredients which may present in plant materials used in aquatic animal diet production.

More studies are needed to identify and isolate active compounds or anti-nutritional components found in *N. nucifera* stem which can be used as natural feed additive for improving growth and feed utilization efficiency in frog rearing.

4.2 Effect on intestinal morphology

It is well known that villi and muscularis of intestines play an important role in the digestion and absorption of nutrients (27,28). In addition, it has been reported that increased muscularis thickness of posterior portion of intestine could be useful for the defecation and reabsorption of water (28). In this present research, we found that anterior and posterior portions of intestine of frog fed the diets mixed with NNSE had significantly higher villi heights, villi widths as well as the thickness of muscularis than the control diet. These results could indicate the mechanism of action of NNSE on frog digestibility and nutrient utilization efficiency (27,28). The reasons for increased villi heights, villi widths and muscularis thickness produced by NNSE are unclear. We hypothesized that medicinal plants and their active

compounds would modulate DNA, RNA and protein synthesis in intestinal epithelial cells and intestinal muscles of frog, resulting in an increase in all these parameters (29,30). In addition, phytochemical ingredients could increase proliferation and mitotic division of intestinal cells in order to improve digestion and absorption of essential nutrients. These results would lead to the enhanced growth performance of frog (29,30).

4.3 Observations on external appearance and feed acceptability

During the treatment period, NNSE supplementation did not affect feeding behavior, feed acceptability and general behavior of frog. External appearances of frog fed the diets containing various levels of NNSE were the same as frog fed the control diet. Thus, our observations indicated that NNSE is safe in a short term use. However, the evaluation of long-term use of NNSE in frog diet should be performed.

5. Conclusion

Our study is the first report to support the use of NNSE as a natural feed additive in the diet to improve growth performance, feed efficiency and intestinal histology of common lowland frog. The suitable level of NNSE for use in the frog diet observed in this study was 5%.

6. Acknowledgement

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7. References

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