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Dietary Supplementation with *Limnophila aromatica* Extract on Growth Performance and Physiological Responses in *Rana rugulosa*

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Abstract

This research was examined the effects of dietary *Limnophila aromatica* extract (LAE) on growth, intestinal histology, hematology and serum biochemistry of common lowland frog (*Rana rugulosa*). Frog (average initial weight 16.00±1.00 g) were fed with the diets supplemented with LAE at the concentrations of 0 (control), 1, 3 and 5% daily for 8 weeks. The qualitative test analysis for phytochemicals showed the presence of glycosides, steroids, alkaloids, flavonoids, phenolic compounds, saponins, terpenoids, coumarins and anthraquinones. After the feeding trial, frog fed different LAE diets significantly increased growth indices and feed conversion efficiency compared with the basal control (P<0.05). The survival rate did not differ among the groups (P>0.05). The improvement in intestinal morphology was observed in frog fed the diets containing LAE compared with the control (P<0.05). White blood cells were significantly decreased in frog fed 5% dietary LAE (P<0.05). There were no significant changes in red blood cells, hemoglobin, hematocrit, mean cell volume, mean cellular hemoglobin and mean cell hemoglobin concentration among the groups (P>0.05). Glucose, cholesterol, triglyceride, and alanine aminotransferase were significantly decreased in the treated frog compared with the control frog (P<0.05). Aspartate aminotransferase, alkaline phosphatase, uric acid, HDL-C, LDL-C, bilirubin-D, bilirubin-T and albumin were not affected by the diet supplementations (P>0.05). This study indicated that LAE supplementation produced a positive effect on growth, intestinal histomorphology and serum biochemistry in frog with the optimal level of 2.66%.

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Introduction

Common lowland frog (*Rana rugulosa*) has long been introduced to commercial frog farming in several parts of Thailand due to high demand for consumption and nutritional values (Thummek et al., 2016). Frogs are generally cultivated in the pond, paddy field, ditch and cage. In 2017, frog production in Thailand was 900 tons with a value of 62 million baht (Fishery Statistics Analysis and Research Group, 2019). The most important markets for frog products are Japan, Taiwan, Hong Kong, USA and European countries (Pariyanonth & Daorerk, 1994). Nowadays, wild populations of frogs have decreased dramatically due to the slow growth rate, habitat destruction, environmental pollution and disease outbreaks (Suriya et al., 2014). According to a high value for their meat, intensive commercial cultures have developed continually to produce higher productive performance (Pariyanonth & Daorerk, 1994). However, these culture methods may increase a high risk of infectious and noninfectious diseases, cannibalism, nutritional imbalance, low water qualities and stress in the cultured frog (Sririkanonda, 2009). Chemicals used in raniculture to promote growth and general health of animals may accumulate in tissues and be a human health hazard (Reverter et al., 2014). Thus, new treatments and preventative procedures, notably, herbal plants and their ingredients have markedly decreased chemical treatments elsewhere.

Medicinal plants and their components are generally used as feed ingredients in raniculture in an attempt to enhance growth and health, feed palatability and attractiveness and treat infectious diseases (Kaewtapee et al., 2011; Serrano et al., 2012; Thainum & Chitmanat, 2019). Scientific reports also support the growth-enhancing effects of the diets supplemented with medicinal plant extracts in a wide variety of frog species (Thainum & Chitmanat, 2019; Kamatit et al., 2016). Kamatit et al. (2016) reported that common lowland frog fed the diets supplemented with waterlily stamen at different levels showed a significant increase in growth parameters and intestinal histology. Thainum & Chitmanat (2019) found that the diets incorporated with *Curcuma longa* extract significantly promoted frog growth, while the diets supplemented with *Pueraria mirifica* increased immune status and resistance against *Aeromonas hydrophila.* Also, Thummek et al. (2016) demonstrated that growth performance and intestinal villi height were significantly enhanced in frog fed the diets containing lotus stamen extract.

Limnophila aromatica is an edible indigenous plant that grows usually in flooded paddy fields of Southeast Asia (Wanyo et al., 2018). Phytochemicals found in *L. aromatica* were terpenoids, flavonoids, phenolic compounds and volatile oils (Bui et al., 2004; Nanasombat & Teckchuen, 2009; Thongdon-A et al., 2013). Nevadensin, gardenin B, isothymusin and pilosin were also isolated and identified in *L. aromatica* (Bui et al., 2004). A nutritional evaluation revealed that *L. aromatica* contains 0.3 g of fat, 7.4 g of carbohydrate, 1.5 g of protein and 1.5 g of fiber (Nutrition Division, 2001). Previous reports suggested that *L. aromatica* has become attractive as health food products because of its antioxidant, antimicrobial, anticancer and vascular protective properties (Kukongviriyapan et al., 2007; Nanasombat & Teckchuen, 2009; Thongdon-A et al., 2013; Wanyo et al., 2018).

Due to an increase in raising aquaculture animals in chemical-free practices, novel natural dietary additives as alternatives for synthetic compounds have now started to receive attention (Chakraborty et al., 2014; Reverter et al., 2014; Munglue, 2015). Recently, scientific data indicated that the diets supplemented with *L. aromatica* produced a significant improvement in growth rates and intestinal morphology of hybrid catfish without negative effects (Munglue et al., 2019b). However, no report is available on the effects of *L. aromatica* extract (LAE) on growth performance and physiological responses in the frog. Therefore, the objectives of this research were conducted to examine the effects of dietary LAE on the growth performance, intestinal histology, hematology and serum chemistry of common lowland frog.

Materials and methods

1. Plant collection and extraction

Arial parts (stem and leaf) of *L. aromatica* were collected from the local garden at Sirindhorn subdistrict, Ubon Ratchathani, Thailand, during the rainy season. The plant specimen was authenticated and herbarium No. Munglue 003 was kept at the Program of Biology, Faculty of Science, Ubon Ratchathani Rajabhat University. The plant samples were cleaned by using deionized water, cut into small parts and macerated with 70% ethanol for 14 days. The extract was then filtrated and evaporated by using a rotary evaporator until dry. The yield was 9.50±0.20% based on dried plant weight.

2. Preliminary phytochemical analysis

Phytochemicals including glycosides, steroids, alkaloids, flavonoids, phenolic compounds, saponins, terpenoids, coumarins, anthraquinones and tannins were detected in LAE by using the standard methods (Evans et al., 2002). The qualitative test analysis of each compound of in LAE was indicated as presence (+) or absence (-) (Munglue et al., 2019a).

3. Diet preparations

The frog diets (Nutrena No. 8211) containing 34% protein and 4% lipid were obtained from the local frog feed company (Cargill Siam Limited, Thailand) and mixed with LAE at concentrations of 0 (control), 1, 3 and 5% by using cassava starch as a binder. The diets were moistened and extruded by using a mincer. The diets were dried in a hot air oven at 60°C for 2 days and subsequently kept in the zip lock bags for further study. Proximate composition was determined using standard methods (AOAC., 2012), as shown in Table 1.

Table 1 Proximate composition of the experimental diets

Parameters	The levels of LAE in the experimental diets (%)			
	0	1	3	5
Moisture	8.65	5.45	4.91	4.21
Ash	12.52	12.71	12.91	12.95
Crude protein	35.35	35.34	35.44	35.41
Crude lipid	7.55	6.39	7.20	7.25

4. Frog preparations

In this present study, animal procedures were approved by Ubon Ratchathani Rajabhat University Animal Care and Use Committee (Approval no. 60003). Frog (average initial weight 16.00±1.00 g) were obtained from Ubon Ratchathani Fishery Cooperation and acclimatized under laboratory conditions for 2 weeks. Frogs were then randomly distributed into 4 treatments with 3 replications (20 frogs per replicate tank). They were cultured in the circular concrete tanks (90 cm in diameter and 50 cm in height) containing dechlorinated water (10 cm height) and covered with black shade nets. Frogs were fed with the experimental diets on Styrofoam for 8 weeks. Water qualities were maintained in the standard conditions for frog culture. The survival was determined throughout the feeding trial.

5. Effects on growth and survival

After 8 weeks of the experimental period, 4 frogs from each replicate tank were randomly collected and weighed. Growth parameters including weight gain (WG), specific growth rate (SGR), average daily gain (ADG), feed conversion ratio (FCR), feed conversion efficiency (FCE) and survival rate (SR) were calculated as follows:

WG (g) = final weight (g) – initial weight (g)

SGR (%/day) = [(*In* final weight (g) – *In* initial weight (g)) /number of experimental days] × 100

ADG (g/day) = final weight/experimental days

FCR = consumed diets (g)/WG (g)

FCE = WG (g)/consumed diets (g)

SR (%) = (final number of frog/initial number of frog) × 100

6. Effects on organosomatic indices

After 8 weeks of the experimental trial, the frogs were fasted for 24 h. Four frogs from each replicate tank were then randomly collected, weighted and double-pithed with a needle. The abdominal wall was carefully opened. Internal organs including liver, intestines, intraperitoneal fat, kidney, spleen and heart were removed, cleared from adjacent tissues, weighted and calculated to obtain the hepatosomatic index (HSI), intestinosomatic index (ISI), intraperitoneal fat (IPF), renosomatic index (RSI), spleen somatic index (SSI) and cardiosomatic index (CSI) using the following equations:

$$\text{Organosomatic indices} = [\text{organ weight (g)}/\text{body weight (g)}] \times 100$$

7. Effects on intestinal histology

The samples of the intestines obtained from two frogs per replicate tank were cleaned using 0.90% normal saline and divided into proximal, middle and distal portions. They were cut into 5 mm transversely and fixed in 10% neutral buffered formalin. The intestinal samples were dehydrated, cleared and embedded in paraffin blocks. Transverse sections were cut into 5 µm and mounted on glass slides, dried and stained in hematoxylin and eosin (H & E). Macromorphology and micromorphology of the intestines were evaluated using a light microscope connected with a computer running Dino Capture 2.0 software (Munglue et al., 2019a). Area of absorption was determined as follows (Abdel-Tawwab et al., 2018):

$$\text{Area of absorption (cm}^2\text{)} = \text{villi height (cm)} \times \text{villi width (cm)}$$

8. Effects on hematology

At the end of the study, blood samples were collected by cardiac puncture from a double-pithed frog and transferred into heparinized tubes for hematological determinations. Hematological parameters including

white blood cells (WBCs), red blood cell (RBCs), hemoglobin (Hb), hematocrit (Hct), mean cell volume (MCV), mean cellular hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were measured using the standard methods of Campbell & Ellis (2007).

9. Effects on serum biochemical values

The blood samples were allowed to clot at 4°C for 3 h and centrifuged at 5000×g for 10 min at room temperature to collect serum. Glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), uric acid, cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), direct bilirubin (bilirubin-D), total bilirubin (bilirubin-T) and albumin were analysed by using commercial test kits obtained from Erba Lachema s.r.o., Czech Republic.

10. Data analysis

Complete Randomized Design (CRD) was used in this research. The results are indicated as the mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA). Duncan test was used when significant differences were observed among the treatments. A significant level of $P<0.05$ was used throughout the experiment. The optimal level of LAE observed in this study was evaluated by using the second order polynomial regression model (Gabriel et al., 2019a).

Results and discussion

1. Preliminary phytochemical analysis

It was exhibited that glycosides, steroids, alkaloids, flavonoids, phenolic compounds, saponins, terpenoids, coumarins and anthraquinones were detected in LAE. However, tannins were absent as demonstrated in Table 2. It was postulated that phytochemicals found in herbal plants have been reported to have growth-promoting effects in several species of aquatic animals (Serrano et al., 2012; Chakraborty et al., 2014; Gabriel et al., 2019a). Also, antioxidant, anti-inflammatory, antibacterial, anti-fungal and antiviral potentials of medicinal plants have been recognized (Reverter et al., 2014; Abdel-Tawwab et al., 2018). Therefore, phytochemicals such as alkaloids, flavonoids, phenolic compounds and terpenoids found in LAE could be responsible for the improvement in growth performance, feed utilization and general health of the cultured frog.

Table 2 Preliminary phytochemical screening of LAE

Phytochemicals	Test results
Alkaloids	+
Flavonoids	+
Terpenoids	+
Phenolic compounds	+
Tannins	-
Steroids	+
Saponins	+
Coumarins	+
Glycosides	+
Anthraquinones	+

Remark: + = presence, - = absence, LAE = *L. aromatica* extract.

Table 3 Growth performance and survival of common lowland frog fed LAE containing diets for 8 weeks

Parameters	Treatments			
	Control	1% LAE	3% LAE	5% LAE
IW (g)^{ns}	15.91±0.39	15.44±0.33	16.00±0.30	16.88±0.84
FW (g)	118.00±7.99^{b}	150.55±7.97^{a}	150.77±7.95^{a}	132.22±7.74^{ab}
WG (g)	102.08±8.06^{b}	135.11±7.94^{a}	134.77±7.96^{a}	115.33±7.50^{ab}
SGR (%/day)	3.55±0.13^{c}	4.04±0.96^{a}	3.98±0.10^{ab}	3.66±0.11^{bc}
ADG (g/d)	1.82±0.14^{b}	2.41±0.14^{a}	2.40±0.14^{a}	2.05±0.13^{ab}
FCR	4.59±0.35^{a}	3.46±0.20^{b}	3.47±0.20^{b}	4.07±0.26^{ab}
FCE	0.22±0.01^{b}	0.29±0.01^{a}	0.30±0.02^{a}	0.25±0.01^{ab}
SR (%)^{ns}	95.00±2.88	95.00±2.88	96.66±1.66	96.66±1.66

Remark: Data are presented as mean ± SEM; Different superscripts^{a-c} in each row are significantly different ($P<0.05$). ns is not significantly different ($P>0.05$). LAE = *L. aromatica* extract; IW = initial weight (g); FW = final weight (g); WG = weight gain (g); SGR = specific growth rate (%/day); ADG = average daily gain (g/day); FCR = feed conversion ratio; FCE = feed conversion efficiency; SR = survival rate (%).

2. Effects on growth and survival

The effects of dietary LAE on growth and survival of frog are presented in Table 3. The results showed that final weight, WG, SGR, ADG and FCE of frog fed the diets supplemented with 1 and 3% LAE were significantly enhanced, whereas FCR values were significantly decreased when compared with the control diet ($P<0.05$). No significant differences were observed in the SR values among the groups ($P>0.05$). It was observed that dietary supplementation with 5% LAE caused the reductions in the growth of cultured frog but these changes did not reach a significant level when compared to the control ($P>0.05$). It is well known that intensive and semi-intensive ranicultures are now faced with poor growth rates and disease outbreaks (Sririkanonda, 2009). Recently, several studies have been performed to examine the effects of natural feed additives on the growth of aquatic animals because many countries have been banded to import aquaculture products treated

with synthetic compounds (Choshasee et al., 2010; Munglue, 2015; Thainum & Chitmanat, 2019). Kamatit et al. (2016) found that dietary supplementation of waterlily stamen extract at concentrations of 3 and 5% significantly improved the growth performance of frog compared with the control. The application of *Pueraria mirifica* power (20 g/kg) or *Butea superba* power (20 g/kg) to the diets promoted the growth of tadpole and also enhanced the metamorphosis in this species (Kaewtapee et al., 2011). *L. aromatica* has been reported to have numerous biological activities because it contains several phytochemical constituents (Bui et al., 2004; Wanyo et al., 2018). The reasons for the growth-promoting effects of LAE could be due to the improvement in feed intake, digestive enzyme production, nutrient metabolism and general health of frog by its phytochemical ingredients such as alkaloids, flavonoids and terpenoids (Chakraborty et al., 2014; Munglue, 2015; Adeshina et al., 2019; Gabriel et al., 2019a). However, LAE supplementation at a high dose (over 3%) could enhance energy utilization to support metabolism, leading to a decrease in frog growth (Tan et al., 2017). Therefore, by using the second order polynomial regression model (Fig. 1), the recommended level of LAE in frog diets to enhance growth rates was 2.66% ($Y = -4.59x^2 + 24.38x$, $R^2 = 0.22$, $P = 0.029$).

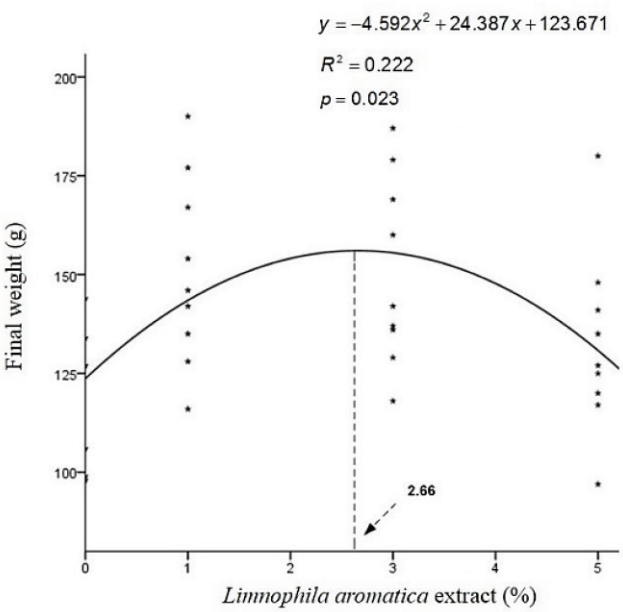

Fig. 1 The second order polynomial analysis on final weight of common lowland frog fed *L. aromatica* extract supplementation for 8 weeks.

3. Effects on organosomatic indices

As shown in Table 4, ISI and RSI of frog fed 5% LAE containing diet were significantly decreased compared with the control ($P<0.05$). There were no significant differences in the HSI, IPF, SSI and CSI of frog fed LAE -supplemented diets compared with the basal diet ($P>0.05$). Changes in the relative organ weights could be useful for indicating the nutritional status, physiological aspects and general health of animals (Schlenk et al., 2008). Decreased body weight due to a reduction of HSI, RSI and ISI are generally associated with lower feed intake, poor metabolism, toxins and anti-nutritional factors found in the diets. Also, low palatability and attractiveness of diets are related to the poor growth performance (Abdel-Haeid, 2007; Tan et al., 2017). In this study, frogs that were fed a diet supplemented with 5% LAE showed a significant decrease in ISI and RSI when compared with the control. Thus, these results also suggest that at a high level of LAE could produce a negative effect on organosomatic indices in the cultured frog.

Table 4 Organosomatic indices of common lowland frog fed LAE containing diets for 8 weeks

Parameters	Treatments			
	Control	1% LAE	3% LAE	5% LAE
HSI (%)^{ns}	6.77±0.59	6.16±0.50	6.12±0.38	5.75±0.53
ISI (%)	2.33±0.18^{a}	2.18±0.10^{ab}	2.02±0.16^{ab}	1.83±0.09^{b}
IPF (%)^{ns}	5.87±0.29	5.52±0.25	5.55±0.20	5.74±0.47
RSI (%)	0.62±0.09^{a}	0.42±0.04^{b}	0.47±0.02^{ab}	0.38±0.06^{b}
SSI (%)^{ns}	0.07±0.01	0.06±0.00	0.06±0.00	0.13±0.04
CSI (%)^{ns}	0.64±0.03	0.83±0.08	0.82±0.10	0.67±0.05

Remark: Data are presented as mean±SEM; Different superscripts^{a-b} in each row are significantly different ($P<0.05$). ns is not significantly different ($P>0.05$). LAE = *L. aromatica* extract; HSI = hepatosomatic index (%); ISI = intestinosomatic index (%); IPF = intraperitoneal fat (%); RSI = renosomatic index (%); SSI = spleen somatic index (%); CSI = cardiosomatic index (%).

4. Effects on intestinal macromorphology

The effects of dietary LAE on intestinal macromorphology of frog are revealed in Fig. 2 and data summarized in Table 5. In the proximal part of the intestine (Figs. 2A–2D), villi height, villi width, inner circulatory smooth muscle and outer longitudinal smooth muscle of frog fed the diets supplemented with LAE were significantly enhanced when compared with frog fed the basal diet ($P<0.05$). In the middle part of the intestine (Figs. 2E–2H), frog fed the diets mixed with LAE significantly increased in villi height, villi width and inner circulatory smooth muscle thickness when

Table 5 Intestinal macromorphology of common lowland frog fed the diets containing LAE for 8 weeks

Intestinal segments	Treatments			
	Control	1% LAE	3% LAE	5% LAE
Proximal				
Villi height (mm)	2.75±0.25^{b}	2.22±0.06^{b}	3.45±0.25^{a}	3.40±0.28^{a}
Villi width (mm)	0.24±0.02^{b}	0.37±0.02^{a}	0.33±0.02^{a}	0.31±0.01^{a}
Inner circulatory muscle thickness (mm)	0.08±0.00^{bc}	0.06±0.00^{c}	0.12±0.01^{a}	0.10±0.00^{ab}
Outer longitudinal muscle thickness (mm)	0.25±0.03^{b}	0.23±0.01^{b}	0.66±0.06^{a}	0.62±0.04^{a}
Middle				
Villi height (mm)	1.45±0.16^{c}	3.67±0.37^{a}	3.73±0.27^{a}	2.75±0.38^{b}
Villi width (mm)	0.19±0.01^{b}	0.33±0.01^{a}	0.30±0.01^{a}	0.31±0.01^{a}
Inner circulatory muscle thickness (mm)^{ns}	0.07±0.00	0.07±0.00	0.06±0.00	0.07±0.00
Outer longitudinal muscle thickness (mm)	0.10±0.01^{b}	0.51±0.04^{a}	0.45±0.04^{a}	0.43±0.04^{a}
Distal				
Villi height (mm)	1.52±0.08^{c}	3.25±0.35^{a}	2.55±0.29^{ab}	2.28±0.28^{b}
Villi width (mm)	0.26±0.02^{c}	0.29±0.01^{bc}	0.36±0.01^{a}	0.33±0.03^{ab}
Inner circulatory muscle thickness (mm)	0.10±0.01^{b}	0.07±0.00^{b}	0.08±0.00^{b}	0.31±0.02^{a}
Outer longitudinal muscle thickness (mm)	0.23±0.04^{c}	0.39±0.01^{b}	0.48±0.03^{a}	0.17±0.02^{c}

Remark: Data are presented as mean±SEM; Different superscripts^{a-c} in each row are significantly different (P<0.05). ns is not significantly different (P>0.05). LAE = *L. aromatica* extract.

compared with the control (P<0.05). No significant difference was observed in the outer longitudinal smooth muscle among the groups (P>0.05). In the distal part of the intestines (Figs. 2I – 2L), villi height, villi width, inner circulatory smooth muscle and outer longitudinal smooth muscle of the experimental frog were significantly increased when compared with the control (P<0.05). Likewise, frog fed LAE supplemented diets had significantly improved absorptive areas compared to those fed the basal diet (P<0.05) (Fig. 3).

These results agree with those by Thummek et al. (2016) who revealed that frog fed the diets mixed with *Nelumbo nucifera* stamen extract showed a significant increase in villi height and villi width when compared to the control. Moreover, Kamatit et al. (2016) also found that the diets supplemented with waterlily stamen extract enhanced villi height and villi width in the frog. The structure of intestinal villi is affected by diet types, digestion processes and toxins (Adeshina et al., 2019; Zeppenfeld et al., 2016). Increased villi height and width would enhance the intestinal absorptive area in the frog (Abdel-Tawwab et al., 2018). The reasons for the increase in villi height, villi width and absorptive area in the present study may be due to the enhancement of cell turn

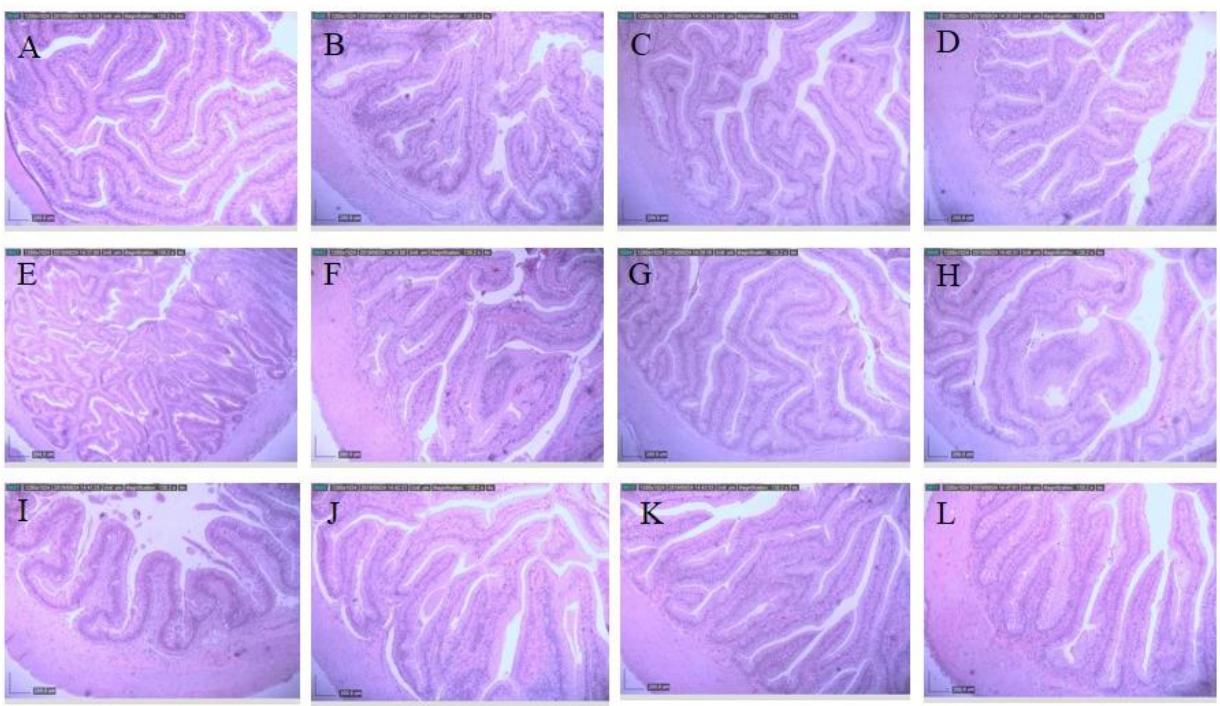

Fig. 2 Effects of dietary supplementation with *L. aromatica* extract on proximal (A-D), middle (E-H), and distal (I-H) parts of the intestines in common lowland frog. Scale bar = 200 μm.

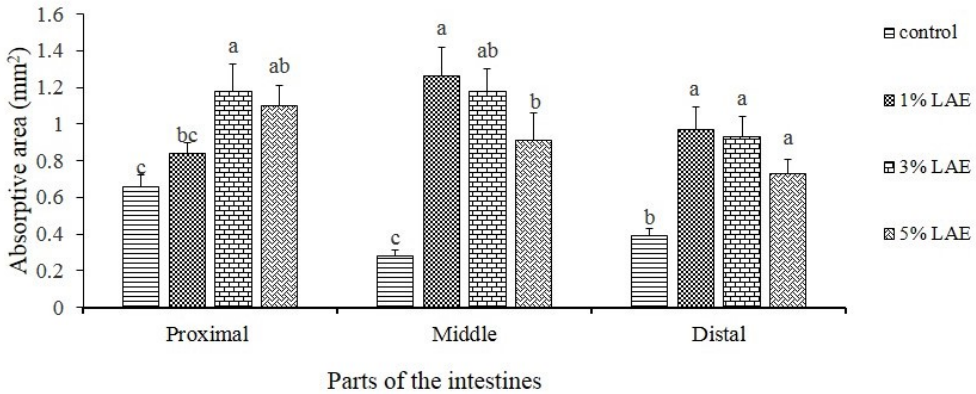

Fig. 3 Effects of dietary supplementation with *L. aromatica* extract on the absorptive area in common lowland frog. Data are presented as mean ± SEM; Different superscripts[a-c] above a bar show a significant difference between treatments (P<0.05). LAE = *L. aromatica* extract.

over rate by LAE in the intestines, leading to the improvements of the growth and feed utilization in the frog (Crosnier et al., 2006).

Functions of muscular layers are to support digestive processes and water retention from the digested contents (Eroschenko, 2008). Decreased muscular thicknesses could reduce the intestinal movement and extend the duration of chyme in the gut lumen (Azizia et al., 2014). This study demonstrated that frog fed the diets supplemented with LAE significantly increased the thicknesses of intestinal muscularis. It has been indicated dietary supplementation with waterlily stamen extract caused a significant increase in the muscular thickness of frog intestine (Kamatit et al., 2016). The increase in the intestinal muscle layer in the present study might be attributed to the improvement of DNA, RNA and protein synthesis by bioactive compounds found in the plant extract (Aanyu et al., 2018; Villasante et al., 2016). Therefore, these results indicate the beneficial effects of LAE on intestinal histomorphology in the frog (Munglue et al., 2019a).

5. Effects on intestinal micromorphology

The effects of dietary LAE on intestinal micromorphology of frog are summarized in Table 6 and the effects on goblet cell number and microvilli height are demonstrated in Fig. 4(A) and 4(B), respectively. In all parts of the intestines, frog fed the experimental diets were significantly increased in enterocyte height, supranucleus height, subnucleus height, goblet cell and microvilli when compared with the control (P<0.05).

Table 6 Intestinal micromorphology of common lowland frog fed the diets containing LAE for 8 weeks.

Intestinal segments	Treatments			
	Control	1% LAE	3% LAE	5% LAE
Proximal				
Enterocyte height (µm)	55.09±2.85^{c}	117.69±4.50^{a}	122.05±3.25^{a}	93.41±7.13^{b}
Supranucleus height (µm)	10.42±0.28^{c}	23.26±0.41^{a}	19.84±0.59^{b}	22.99±0.72^{a}
Subnucleus height (µm)	10.35±0.37^{c}	19.71±0.55^{a}	17.16±0.79^{b}	21.32±0.72^{a}
Middle				
Enterocyte height (µm)	82.41±5.05^{c}	134.16±4.59^{a}	131.62±2.87^{a}	115.78±3.36^{b}
Supranucleus height (µm)	14.59±0.46^{c}	18.88±0.79^{b}	19.37±0.49^{b}	21.52±1.01^{a}
Subnucleus height (µm)	11.57±0.55^{c}	18.58±0.66^{a}	18.84±0.55^{a}	16.36±0.73^{b}
Distal				
Enterocyte height (µm)	91.53±4.60^{b}	122.18±3.78^{a}	126.52±3.42^{a}	116.01±2.23^{a}
Supranucleus height (µm)	13.88±0.74^{b}	22.88±0.49^{a}	21.19±0.93^{a}	21.79±0.68^{a}
Subnucleus height (µm)	10.32±0.56^{b}	16.61±0.80^{a}	18.43±0.87^{a}	17.22±0.65^{a}

Remark: Data are presented as mean±SEM; Different superscripts[a-c] in each row are significantly different (P<0.05). LAE = *L. aromatica* extract.

It is well established that enterocyte plays a key role in the production of digestive enzymes and absorption of essential nutrients (Eroschenko, 2008). Goblet cells are located between enterocytes and play an important role in the production of mucus for covering the apical surface of enterocytes (Bakke et al., 2011). Microvilli are served as the areas for nutrient metabolism and utilization (Dimitroglou et al., 2010). Significant increases in enterocyte height, supranucleus height, subnucleus height, goblet cell and microvilli height were observed in frog fed the diets containing LAE. Similarly, a study by Munglue et al. (2019a) reported that hybrid catfish fed a mixture of lasia extract significantly increased goblet cell number and microvilli height. Also, the application of mannan oligosaccharide (MOS) to the diets at the concentrations of 0.2 and 0.4%

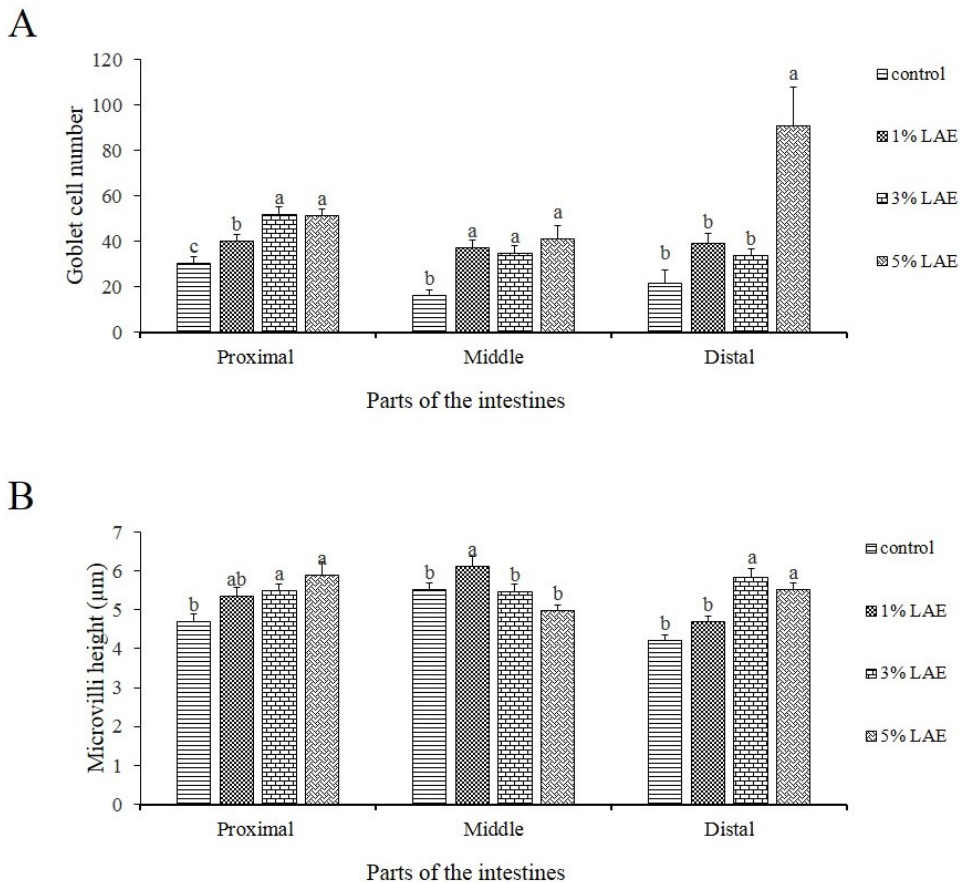

Fig. 4 Effects of dietary supplementation with *L. aromatica* extract on (A) microvilli height and (B) goblet cell number in common lowland frog. Data are presented as mean ± SEM; Different superscripts[a-c] above a bar show a significant difference between treatments (P<0.05). LAE = *L. aromatica* extract.

significantly increased microvilli height and microvilli density in the proximal and distal intestines of gilthead sea bream (*Sparus aurata*) (Dimitroglou et al., 2010). The enhancement of enterocyte, goblet cell and microvilli height by LAE might be due to its chemical compounds that enhanced the division of regenerative cells to replace several cell types in the villi (Antushevich et al., 2014; Crosnier et al., 2006).

6. Effects on hematology

Table 7 summarizes the effects of dietary supplementation of LAE on hematological indices of the frog. At the end of 8 weeks, it was found that frog fed 5% LAE supplemented diet significantly decreased in WBCs contents when compared with the control (P<0.05). No significant differences were observed in RBCs, Hb, Hct, MCV, MCH and MCHC among the groups (P>0.05).

Hematological studies could be useful for the evaluation of general health status, gene mutation and

Table 7 Hematological values of common lowland frog fed the diets containing LAE for 8 weeks.

Parameters	Treatments			
	Control	1% LAE	3% LAE	5% LAE
WBCs ($\times 10^4$ cell/mm^3)	8.95±0.10^{a}	8.39±0.19^{ab}	8.86±0.14^{ab}	8.24±3.82^{b}
RBCs ($\times 10^{11}$ cell/l)^{ns}	1.58±0.11	1.40±0.09	1.51±0.07	1.48±0.01
Hb (g/dl)	12.34±0.51^{ab}	11.88±0.44^{b}	13.86±0.65^{a}	11.42±0.70^{b}
Hct (%)^{ns}	30.07±2.26	26.11±2.26	27.55±1.84	27.88±3.27
MCV (fl)^{ns}	182.09±5.62	185.57±3.91	182.99±5.22	184.04±4.47
MCH (pg)^{ns}	839.01±68.98	870.08±48.03	919.05±59.06	836.59±84.33
MCHC (g/dl)^{ns}	473.12±51.40	474.56±33.73	512.82±48.96	464.35±55.18

Remark: Data are presented as mean±SEM; Different superscripts^{a-b} in each row are significantly different (P<0.05). ns is not significantly different (P>0.05). LAE = *L. aromatica* extract. WBC = white blood cell ($\times 10^4$ cell/mm^3); RBC = red blood cell ($\times 10^{11}$ cell/l); Hb = hemoglobin (g/dl); Hct = hematocrit (%); MCV = mean corpuscular volume (fl); MCH = mean corpuscular hemoglobin (pg); MCHC = mean corpuscular hemoglobin concentration (g/dl).

physiological pathology in the frog (Schlenk et al., 2008). Factors affecting hematological values of aquatic animals including species, age, sex, temperature, stress, environmental surrounding, toxins and diets have been reported (Carlson & Zelikoff, 2008). Decreased RBCs are related to infectious diseases, malnutrition, toxic exposure, kidney and spleen diseases, stress and hypoxia (Carlson & Zelikoff, 2008). Increased WBCs are commonly used to indicate stress, infection and inflammation in the frog (Anderson, 1996). This study revealed that frog fed the diet supplemented with 5% LAE diet showed a significant decrease in WBCs. In similar study, Gabriel et al. (2019) indicated that fish fed the diets incorporated with 4% *Aloe vera* polysaccharides exhibited a significant decrease in platelet, WBCs and granular leukocytes. It is suggested that a high dose of LAE may impair the physiological functions of hematopoietic organs by some phytochemicals (Anderson, 1996; Gabriel et al., 2019b), resulting in the lower WBCs counts in this study.

7. Effects on serum biochemistry

The effects of dietary supplementation of LAE on serum biochemistry of frog are presented in Table 8. Levels of glucose, cholesterol, triglyceride and ALT of the experimental groups decreased significantly. There were no changes in AST, ALP, uric acid, HDL-C, LDL-C, bilirubin-D and bilirubin-T among the groups.

Table 8 Serum biochemistry of common lowland frog fed the diets containing LAE for 8 weeks.

Parameters	Treatments			
	Control	1% LAE	3% LAE	5% LAE
Glucose (mg/dl)	38.91±1.83^{a}	23.82±1.76^{b}	24.79±2.13^{b}	28.61±1.97^{b}
AST (U/l)^{ns}	394.08±53.48	463.63±120.77	442.86±113.75	602.42±69.03
ALT (U/l)	983.79±118.85^{a}	603.89±114.86^{ab}	482.21±116.77^{b}	843.65±157.58^{ab}
ALP (U/l)^{ns}	64.27±5.21	45.80±19.19	53.92±5.77	43.19±10.27
Uric Acid (mg/dl)^{ns}	58.67±21.61	49.62±11.78	38.69±6.34	33.85±12.34
Cholesterol (mg/dl)	121.03±6.69^{a}	74.31±15.52^{b}	54.73±9.11^{b}	78.59±9.75^{b}
Triglycerides (mg/dl)	32.54±4.38^{a}	29.33±2.76^{ab}	18.5±5.66^{b}	22.18±3.42^{ab}
HDL-C (mg/dl)^{ns}	40.05±4.60	40.21±11.50	69.00±16.71	34.13±2.77
LDL-C (mg/dl)	25.01±8.43^{ab}	32.69±5.64^{b}	39.80±12.71^{a}	22.35±4.04^{ab}
Bilirubin-D (mg/dl)^{ns}	5.64±0.51	9.82±1.32	7.35±1.03	5.50±1.19
Bilirubin-T (mg/dl)^{ns}	6.36±2.20	4.28±0.78	4.42±1.52	6.98±0.32
Albumin (g/dl)^{ns}	5.01±0.57	4.45±0.33	4.58±0.43	3.96±0.77

Remark: Data are presented as mean±SEM; Different superscripts^{a-b} in each row are significantly different (P<0.05). ns is not significantly different (P>0.05). LAE = *L. aromatica* extract; AST = Aspartate aminotransferase (U/l); ALT = alanine aminotransferase (U/l); ALP = alkaline phosphatase (U/l); HDL-C = high density lipoprotein cholesterol (mg/dl); LDL-C = low density lipoprotein (mg/dl).

Glucose is generally used to demonstrate physiological stresses in aquatic animals. Results obtained showed a significant decrease in serum glucose in frog fed the experimental diets which would be associated with hypoglycemic effects of *L. aromatica* (Thongdon-A & Inprakhon, 2009). It is hypothesized that LAE may induce pancreatic β-cells to release insulin, resulting in lower blood glucose contents (Serrano et al., 2012). Additionally, LAE may enhance glycogen synthesis and accumulation in frog hepatocytes (Serrano et al., 2012). Similar results were reported by Abdel-Tawwab et al. (2018) who found that African catfish (*Clarias gariepinus*) fed the diets containing clove basil (*Ocimum gratissimum*) leaf extract (0, 5, 10 and 15 g/kg diet) for 12 weeks significantly decreased blood glucose levels when compared with the control diet.

Triglyceride levels are associated with lipid peroxidation and lipid accumulation in hepatocytes (Zhai et al., 2016). Cholesterol is the main component of the cell membrane and myelin sheath and serves as a precursor for bile acid synthesis (Zhu et al., 2014). Serum cholesterol level is related to lipid metabolism homeostasis (Gabriel et al., 2015; Figueiredo-Silva et al., 2005). Increased cholesterol may indicate the prevalence of hepatitis, kidney diseases, pancreatitis and gall bladder diseases (Marshall et al., 2012). In this research, dietary LAE produced a significant decrease in serum triglyceride and cholesterol in the frog. Similary, Zhai et al. (2016) found that dietary surfactin at 100 and 200 mg/diet decreased triglyceride and cholesterol levels in Nile tilapia fingerlings. Mabe et al. (2018) reported that fish fed the diets mixed with bamboo charcoal at 1, 2 and 4% showed a reduction in cholesterol concentration. A significant decrease in triglyceride by LAE might be attributed to the inhibitory effects of its phytochemical contents on metabolism and utilization of fat *in vivo* and lipid peroxidation in the gut (Sribusarakum et al., 2004). Additionally, it is noted that a decrease in cholesterol levels might be due to the hypocholesterolemic effects of LAE or its phytochemical substances such as flavonoids, saponins and alkaloids in the frog (Kishawy et al., 2016; Serrano et al., 2012).

AST is an indicator for detecting the impairment of the liver, heart, muscle, pancreas and kidney (Coppo et al., 2003; Kumar et al., 2013). Increased AST levels are used to demonstrate infectious, immune or metabolic abnormalities (Coppo et al., 2003). Lower AST level was found in frog fed a mixture of LAE in this research. Gabriel et al. (2019b) reported that dietary *Aloe vera*

polysaccharide caused a significant decrease in AST in African catfish. In this study, decreased AST activities may be related to the hepatoprotective effect of LAE against oxidative stress in the frog (Dadras et al., 2016; Gabriel et al., 2019a; 2019b).

Serum ALT, ALP, uric acid, HDL-C, LDL-C, bilirubin-D and bilirubin-T in the experimental groups were similar to the control. Thus, these findings revealed that LAE did not produce any side effect on the liver, RBCs, skeletal and cardiac muscle, pancreas and kidney in the frog (Coppo et al., 2003; Marshall et al., 2012).

Conclusion

Overall, this is the first report to indicate that dietary supplementation of LAE produced a significant increase in growth indices, feed utilization efficiency and intestinal histology of common lowland frog. Additionally, the reductions in glucose, cholesterol, triglyceride, LDL-C and AST levels showed hypoglycemic, hypocholesterolemic and hepatoprotective properties of LAE in frogs. Based on the second order polynomial analysis, the optimal level of LAE observed in this study was 2.66%. Therefore, the results of this study support the potential use of LAE as a growth-promoting agent in the raniculture industries.

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