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Content

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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% ($Y = -4.59x^2 + 24.38x$, $R^2 = 0.22$, $P = 0.029$).

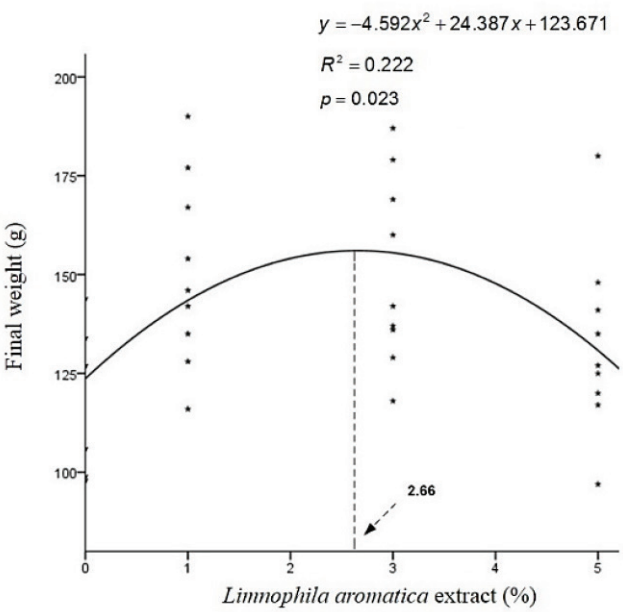

Fig. 1 The second order polynomial analysis on final weight of common lowland frog fed *L. aromatica* extract supplementation for 8 weeks.

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

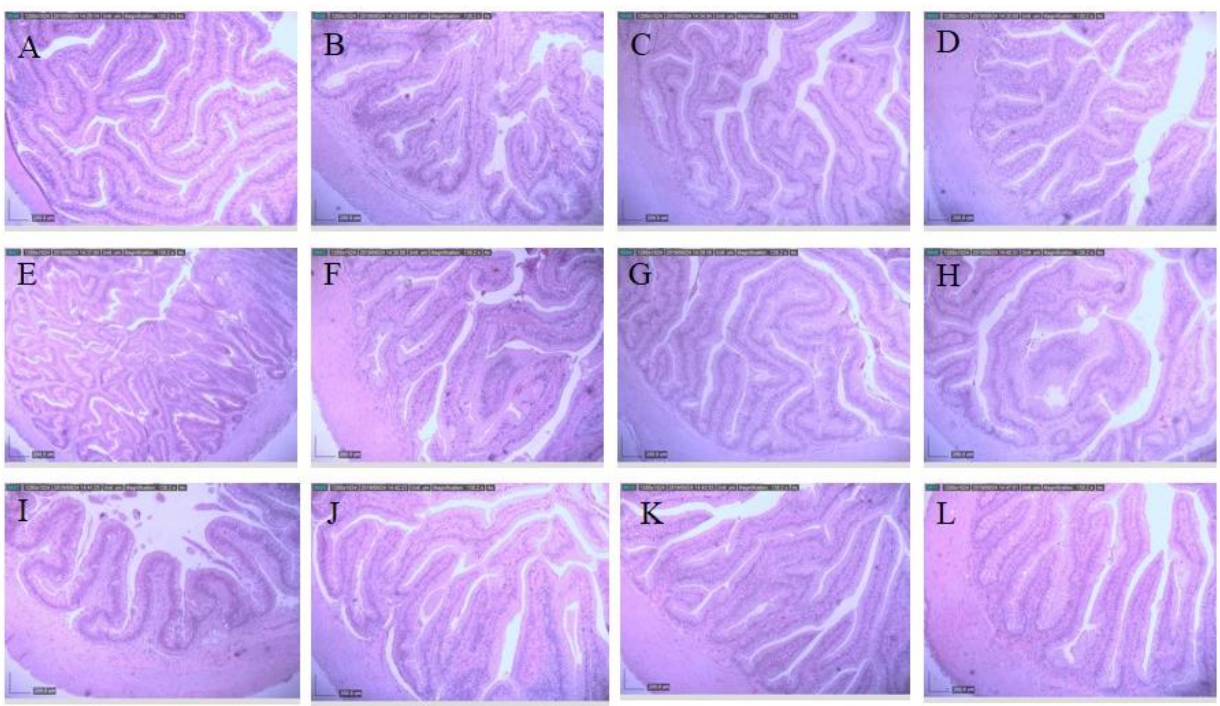

Fig. 2 Effects of dietary supplementation with *L. aromatica* extract on proximal (A-D), middle (E-H), and distal (I-H) parts of the intestines in common lowland frog. Scale bar = 200 μm.

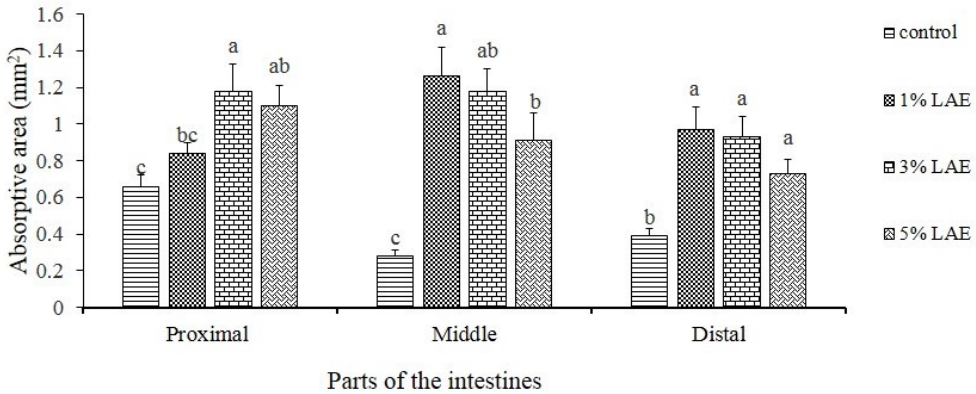

Fig. 3 Effects of dietary supplementation with *L. aromatica* extract on the absorptive area in common lowland frog. Data are presented as mean ± SEM; Different superscripts[a-c] above a bar show a significant difference between treatments (P<0.05). LAE = *L. aromatica* extract.

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%

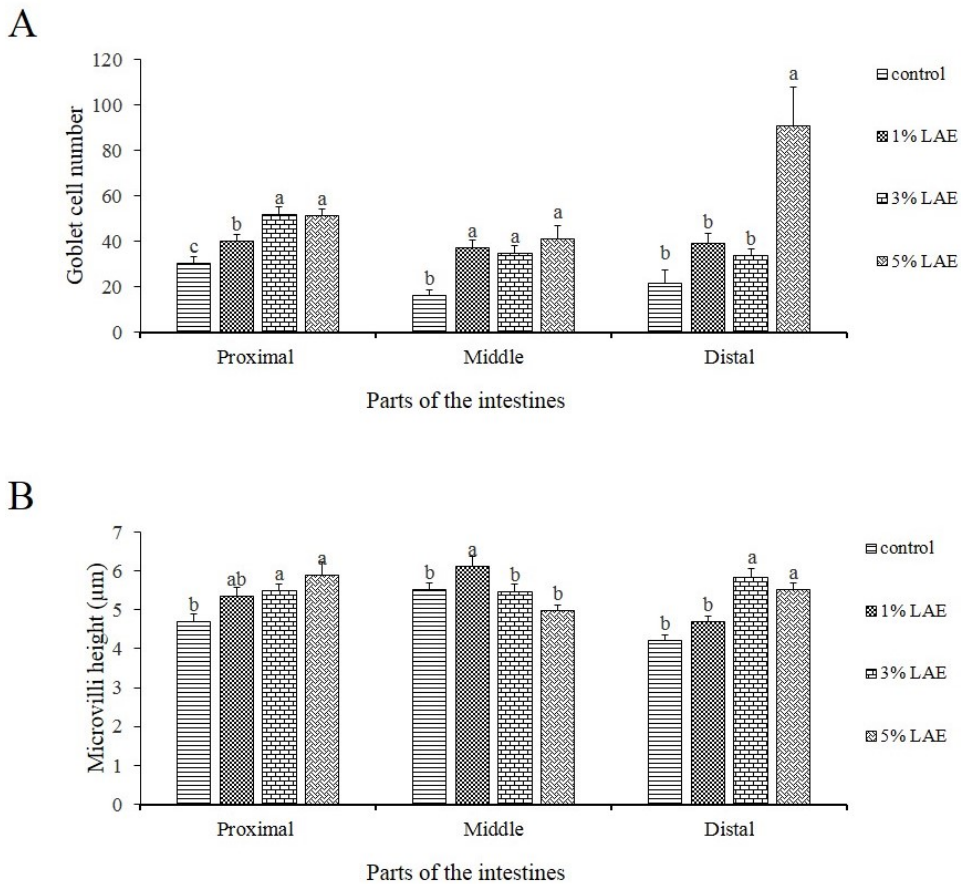

Fig. 4 Effects of dietary supplementation with *L. aromatica* extract on (A) microvilli height and (B) goblet cell number in common lowland frog. Data are presented as mean ± SEM; Different superscripts[a-c] above a bar show a significant difference between treatments (P<0.05). LAE = *L. aromatica* extract.

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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Anti-Cariogenic Activity, Cytotoxicity and Chemical Constituents of *Zingiber rubens* Roxb.

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Abstract

The objectives of this study were to evaluate the antibacterial activity, cytotoxicity and chemical constituents of *Zingiber rubens* Roxb. Four parts of the dried plant materials including rhizomes, stems, leaves and fruits were extracted with distilled water and 95% ethanol to obtain crude extracts. The essential oil was derived from the rhizome using steam distillation. After that, the crude extracts and essential oil were screened for antibacterial activity against *Lactobacillus casei* TISTR 390 and *Streptococcus mutans* ATCC 25175 using the agar disc diffusion and broth dilution methods. Moreover, the cytotoxicity of the plant extracts was evaluated using MTT assay on Vero cells. The results show that all ethanolic extracts could inhibit the tested bacteria with an inhibition zone ranging from 7.00–8.67 mm and MIC/MBC ranging from 6.25–50 mg/ml. Additionally, the essential oil also effectively inhibited both the bacterial strains with an inhibition zone ranging from 8.17–8.83 mm and MIC/MBC of 250 mg/ml. For *in vitro* cytotoxic properties, all plant extracts exhibited no toxicity on Vero cells with CC_{50} between 30.32 - > 1,000 µg/ml. Notably, the essential oil derived from the plant rhizome also revealed no toxicity *in vitro* with CC_{50} of 2.5 µg/ml. Furthermore, the essential oil from the rhizome identified volatile compounds using Gas Chromatography-Mass Spectrometry (GC-MS). Importantly, 2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)- or zerumbone is the main compound in essential oil with a % peak area of 20.47 %. Thus, the crude extract and essential oil of this plant could inhibit cariogenic bacteria and display low toxicity on human cells, which may be useful in the development of an antibacterial agent in the future.

Introduction

Dental caries or tooth decay is a global public health problem. Severe caries often cause pain and infection, resulting in tooth extraction and also have an effect on general health and well–being (Veiga et al., 2016; Yadav & Prakash, 2017). The major cause of tooth decay is the colonization of tooth surfaces by cariogenic bacteria (Costa et al., 2012; Selwitz et al., 2007). Oral Streptococci, especially *Streptococcus mutans,* is one of

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the cariogenic bacteria, most associated with initial formations of caries (Van Houte, 1994). This bacterium synthesizes extracellular polysaccharides from sucrose and produces acids, which eventually demineralizes tooth enamel (Loesche, 1986). Other microflora, such as *Streptococcus sanguis, Actinomyces* and *Lactobacillus* spp., which tolerate acidity can survive and cause severe caries (Hamada & Slade, 1980). Various chemical compounds such as alcohol, fluoride and antibiotics (chlorhexidine, erythromycin, ampicillin, penicillin) have been widely used for dental caries prevention for many years (Baker et al., 1987; Wolinsky, 1994). However, it has some side effects including brown staining of the tooth, alteration in the sensation of taste, soreness in the oral mucosa (Vieira et al., 2014). Importantly, antibiotic-resistant bacterial populations are increasing rapidly. For this reason, antimicrobial agents from natural sources may become another choice for bacterial disease treatment. Medicinal plants have long been used for treatment due to their availability, low cost, no toxic and minimal side effects compared to conventional antibiotics (Abdel-Aziz et al., 2016).

Zingiber rubens Roxb. belonging to the family Zingiberaceae, is a native plant mostly distributed over tropical and subtropical areas including Thailand, Myanmar, Vietnam, India, Bangladesh, China South-Central and East Himalaya (Ahmed, 2008). Various species are widely used as foods, spices, flavoring agents and traditional usages such as herbal drugs for the treatment of carminative, stomachache, diarrhea, stimulant and cold (Nontasit et al., 2015; Yob et al., 2011). Many plants of the Zingiberaceae family have been found to possess antibacterial properties against several pathogens. (Abdul et al., 2008; Habsah et al., 2000; Sanpa & Sanpa, 2019). Nevertheless, few studies have investigated the biological activity of *Z. rubens*. The previous studies showed that the ethanolic extract of this plant exhibited moderate antioxidant activity among other species (Kantayos & Paisooksantivatana, 2012). However, its biological capabilities in other aspects have not been much studied. Therefore, this study evaluates the antibacterial activities against some cariogenic bacteria and cytotoxic properties of crude plant extracts and essential oil of various parts of *Z. rubens*. Moreover, the chemical composition was analyzed using GC-MS analysis.

Materials and methods

1. Plant extraction

Zingiber rubens Roxb. was collected from the local area of Lamphun province, Thailand. For crude plant extraction, four parts of fresh plants including stems, rhizomes, leaves and fruits were washed and dried at 60°C. The ground plants (100 g) were extracted with distilled water at 45°C for 3 hours or macerated with 95% ethanol for 72 hours at room temperature at a proportion 1:10 (w/v). After that, the plant's extracts were filtrated, evaporated under vacuum using rotary evaporator and then lyophilized to obtain crude powder. The crude plant extracts were dissolved using dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml.

2. Essential oil extraction

Fresh rhizome was grounded (50 g) using a blender. The essential oil was obtained by steam distillation at 100°C for 3 hours with a Clevenger - type apparatus. The essential oil was separated from water using sodium sulfate anhydrous (Na_2SO_4) and stored at 4°C for further study.

3. Antibacterial activity

3.1 Agar disc diffusion assay

The antibacterial effect of plant extracts and essential oil was tested against *Lactobacillus casei* TISTR390 and *Streptococcus mutans* ATCC25175 by agar disc diffusion assay according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2015). The tested bacteria were cultured in de Man Rogosa Sharpe broth (MRS broth) (Himedia, India) for *L. casei* and Brain heart infusion broth (BHI broth) (Himedia, India) for *S. mutans* and incubated at 37°C for 18 hours. After that, the turbidity of the bacterial cultures was adjusted to be comparable to McFarland standard No. 0.5 (Himedia, India) to obtain approximately 1.0 x 10^8 CFU/ml. The cultures of bacteria were swabbed on MRS agar for *L. casei* and BHI agar for *S. mutans*. Then, 20 µl of crude extracts (100 mg/ml) and essential oil (1,000 mg/ml) were applied on 6 mm diameter sterile paper discs (Macherey-Nagel®) and the discs were placed on the agar. 100% DMSO was used as control and chlorhexidine at a concentration of 2 mg/ml was used as a positive control. These plates were incubated at 37°C for 24 hours.

3.2 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the plant

extracts and essential oil were determined using broth dilution method with slight modifications of CLSI (2015). The plant extracts, essential oil and chlorhexidine were diluted in growth medium by two-fold serial dilutions into 96-well plate to obtain concentrations between 6.25-100 mg/ml for plant extracts, 250-1,000 mg/ml for essential oil and 0.0039-2 mg/ml for chlorhexidine. Then, the bacterial culture with 1 x 10^8 CFU/ml was inoculated to each well plate and incubated at 37°C for 24 hours. MIC was defined as the lowest concentration of plant extract that prevents the growth of bacterial strains.

For MBC evaluation, the well plate with no visible turbidity in MIC assay were streaked onto medium agar plates and incubated at 37°C for 24 hours. After incubation, MBC was determined as the lowest concentration of the plant extract showing no visible growth of bacterial strains.

4. Cytotoxicity assay

The cytotoxicity assay was conducted on Vero cells (African green monkey kidney cell) using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Yu et al., 2004). The cells in 96-well plates were cultured in Dulbecco's modified eagle medium (D-MEM) (Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, UK) and incubated at 37°C in 5% CO_2 incubator. After incubation, the growth medium was removed and replaced with 2-fold serial dilutions of plant extracts (7.81-1,000 µg/ml) and essential oil (0.078-10 µg/ml). The cell control and vehicle control dimethyl sulfoxide (DMSO) was used as a negative control. The plates were incubated at 37°C in 5% CO_2 for 72 hours. Then, the plant extracts, essential oil and growth medium were removed and the MTT reagent (5 mg/ml) (Bio Basic, Canada) was added and incubated for 4 hours. Finally, the formazan crystal blue was dissolved with 100 % DMSO and the absorbance was measured at 540 and 630 nm using microplate reader (Biochrom, UK). The percentage of viability was calculated comparing to the cell control and 50% cytotoxic dose (CC_{50}) concentration was determined using probit analysis.

5. Chemical composition analysis

The essential oil component analyses were conducted by NSTDA Characterization and Testing Service Center, National Science and Technology Development Agency (NSTDA), Thailand. The constituents of the oil were analyzed using Gas chromatography-Mass spectrometry (GC-MS). The GC-MS analysis was performed on GC-MS TQ8050 (Shimadzu, Japan) equipped with DB-5 MS columns (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technology). The analytical condition was applied by Dai et al., 2013. Helium was used as a carrier gas and was adjusted to column velocity flow of 0.69 ml/min. The injector temperature (PTV) was 250°C, detector temperature 250°C, column temperature-programmed 60°C (2 min hold) to 220°C (10 min hold) at a rate of 4°C/min. One milliliter of diluted oil sample (1:10 v/v in methanol) was injected in the split mode with split ratio 10:1 by auto-injection. Inlet pressure was 30.1 kPa. Identification of the components was achieved based on retention time and mass spectral matching with NIST/EPA/NIH Mass Spectral Library 2014.

6. Statistical analysis

The results of three replicates were reported as mean ± SD. Analysis of variance (ANOVA) was calculated using Duncan's new multiple range test at $P < 0.05$.

Results

1. Antibacterial activity

The antibacterial activity of plant extracts and essential oil were evaluated using agar disc diffusion and broth dilution method. The results were shown in Table 1 and 2. According to disc diffusion assay, all ethanolic extracts and essential oil could inhibit both tested bacteria with the inhibition zones ranging from 7.00-8.83 mm while all aqueous extracts could not inhibit any tested bacteria. However, the zones of inhibition of all plant extracts and the essential oils were smaller than that of the positive control, chlorhexidine. Moreover,

Table 1 Zone of inhibition of medicinal plant extracts against *L. casei* TISTR 390 and *S. mutans* ATCC 25175 by agar disc diffusion method

Extracts	Part used	Zone of inhibition ± SD (mm)	
		L. casei TISTR 390	*S. mutans* ATCC 25175
Aqueous (100 mg/ml)	rhizome	NZ	NZ
	Stem	NZ	NZ
	leave	NZ	NZ
	fruit	NZ	NZ
Ethanol (100 mg/ml)	rhizome	7.17 ± 0.29^{c}	7.67 ± 0.58^{cd}
	Stem	7.17 ± 0.29^{c}	8.67 ± 0.58^{b}
	leave	7.17 ± 0.29^{c}	7.17 ± 0.29^{d}
	fruit	7.00 ± 0.00^{c}	7.00 ± 0.00^{d}
Essential oil (1,000 mg/ml)	rhizome	8.83 ± 0.29^{b}	8.17 ± 0.29^{bc}
chlorhexidine (2 mg/ml)	-	27.67 ± 0.58^{a}	36.00 ± 0.00^{a}
DMSO (100%)	-	NZ	NZ

Remark: NZ; no zone of inhibition, Means with different letters^{a-d} in each column are significant differences ($p<0.05$) for each extract

the plant extracts which inhibited the tested bacteria were further evaluated for MIC and MBC values. Table 2, shows that the ethanolic extracts of the stem had an effective antibacterial activity with MIC and MBC of 6.25 mg/ml for both bacterial species. Furthermore, the plant essential oil also shows the MIC and MBC of 250 mg/ml on *L. casei* TISTR390 and *S. mutan*s ATCC25175.

Table 2 MIC and MBC values of crude extract and essential oil from *Z. rubens* against *L. casei* TISTR 390 and *S. mutans* ATCC 25175 using broth dilution method

Extracts	Part used	MIC/MBC			
		L. casei TISTR 390		*S. mutans* ATCC 25175	
		MIC	MBC	MIC	MBC
Aqueous (mg/ml)	rhizome	ND	ND	ND	ND
	stem	ND	ND	ND	ND
	leave	ND	ND	ND	ND
	fruit	ND	ND	ND	ND
Ethanol (mg/ml)	rhizome	6.25	6.25	25	25
	stem	6.25	6.25	6.25	6.25
	leave	6.25	6.25	50	50
	fruit	12.5	12.5	50	50
Essential oil (mg/ml)	rhizome	250	250	250	250
Chlorhexidine (mg/ml)	-	1	1	0.0039	0.0039

Remark: ND; not determine.

2. Cytotoxicity of plant extracts

The results of cytotoxicity of crude extracts and essential oil of various parts of *Z. rubens* to the Vero cells are presented in Table 3. For *in vitro* cytotoxic properties, the aqueous extract exhibits lower toxicity than ethanolic extract with CC_{50} > 1,000 µg/ml while the CC_{50} concentrations of ethanolic extract is between 30.32 - 377.35 µg/ml. However, all extracts show a CC_{50} more than 20 µg/ml, which is considered to be nontoxic as regarded by the US National Cancer Institute (NCI).

3. Chemical constituents of essential oil

The yield of essential oil obtained from rhizomes by steam distillation was 0.21 % (w/w) with yellow color and an agreeable smell. Eighty-seven constituents identified by GC-MS analysis are represented in Table 4. The major constituents of oils are 2,6, 10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-, commonly known as zerumbone (20.47%), 1,4,7,-cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z- (7.31%), cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)- (6.90%), (1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo [4.4.0.02,7] decane-rel- (6.12%) and isospathulenol (4.11%).

Table 3 Cytotoxicity of crude extracts and essential oil of *Z. rubens* to the Vero cell

Extracts	Part used	50% cytotoxic concentration, CC_{50}
Aqueous	Rhizome	> 1,000 µg/ml
	Stem	> 1,000 µg/ml
	Leave	> 1,000 µg/ml
	Fruit	> 1,000 µg/ml
Ethanolic	Rhizome	30.32 µg/ml
	Stem	66.66 µg/ml
	Leave	377.35 µg/ml
	Fruit	147.58 µg/ml
Essential oil	Rhizome	2.50 µg/ml

Table 4 The main components and peak-area percentage (%) of essential oil of *Z. rubens*

Peak	RT (min)	% Area	Identification of the compounds
1	6.968	0.07	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
2	7.220	0.95	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3	7.732	0.90	Camphene
4	8.398	0.12	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
5	8.624	2.67	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
6	8.863	0.35	.beta.-Myrcene
7	9.511	2.12	.alpha.-Phellandrene
8	9.576	0.68	Santolina triene
9	9.818	0.08	(+)-4-Carene
10	10.088	0.88	o-Cymene
11	10.248	0.61	D-Limonene
12	10.363	0.67	Eucalyptol
13	10.767	0.19	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-
14	11.214	0.16	.gamma.-Terpinene
15	12.168	0.26	Cyclohexene, 3-methyl-6-(1-methylethylidene)-
16	12.328	0.05	Fenchone
17	12.637	0.06	Linalool
18	14.437	0.35	(+)-2-Bornanone
19	15.305	0.11	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-
20	15.592	0.10	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-
21	16.107	0.12	.alpha.-Terpineol
22	17.917	0.12	5,8-Decadien-2-one, 5,9-dimethyl-, (E)-
23	19.222	0.05	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-
24	20.724	0.05	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-
25	21.115	6.90	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl) -1-(1-methylethyl)-, (3R-trans)-
26	21.385	0.27	.alpha.-Cubebene
27	22.131	0.07	1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl -5-(1-methylethyl)-, [1S-(1.alpha.,2.alpha.,3a.beta., 4.alpha.,5.alpha.,7a.beta.
28	22.358	0.82	.alfa.-Copaene
29	22.532	0.08	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
30	22.804	1.71	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
31	23.180	0.19	(1S,5S)-2-Methyl-5-((R)-6-methylhept-5-en-2-yl) bicyclo[3.1.0]hex-2-ene
32	23.387	0.30	1H-Cyclopropa[a]naphthalene, 1a, 2, 3, 3a, 4, 5, 6, 7b-octahydro-1,1,3a,7-tetramethyl-, [1aR-(1a.alpha., 3a.alpha.,7b.alpha.)]-
33	23.842	1.72	Caryophyllene
34	24.085	1.02	1,5-Cyclodecadiene, 1,5-dimethyl-8- (1-methylethylidene)-, (E,E)-
35	24.283	0.22	.alpha.-Maaliene
36	24.485	0.52	(1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1, 2, 3, 3a, 6, 8a-hexahydroazulene
37	24.776	1.14	(1S,4S,4aS)-1-Isopropyl-4, 7-dimethyl-1, 2, 3, 4, 4a, 5-hexahydronaphthalene
38	25.135	7.31	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
39	25.638	1.50	.gamma.-Muurolene

Table 4 The main components and peak-area percentage (%) of essential oil of *Z. rubens* (Cont.)

Peak	RT (min)	% Area	Identification of the compounds
40	25.911	6.12	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel-
41	26.183	1.65	Benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl) bis-
42	26.256	1.39	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0] undeca-2,6-diene
43	26.462	0.44	isoledene
44	26.609	0.96	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
45	26.961	0.86	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1.alpha.,4a.beta.,8a.alpha.)]-
46	27.268	079	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-
47	27.506	0.44	Cyclohexane, 1-methyl-2,4-bis(1-methylethenyl)-, (1.alpha.,2.beta.,4.beta.)-
48	27.635	0.10	Selina-3,7(11)-diene
49	27.848	0.27	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha., 4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha.,3.alpha., 4.beta.)]-
50	27.995	0.23	7-epi-cis-sesquisabinene hydrate
51	28.266	3.64	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-
52	28.486	0.06	1,3,12-Nonadecatriene
53	28.729	0.35	Isospathulenol
54	28.913	1.50	Caryophyllene oxide
55	29.032	0.63	Isospathulenol
56	29.444	2.90	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
57	29.56	0.32	Ledol
58	29.776	2.51	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo [9.1.0]dodeca-3,7-diene
59	30.002	0.38	(3R, 3aR, 3bR, 4S,7R, 7aR)-4-Isopropyl-3, 7-dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2] benzen-3-ol
60	30.323	4.41	Isospathulenol
61	30.559	1.68	(-)-Spathulenol
62	30.810	0.75	Eudesma-4(15),7-dien-1.beta. -ol
63	31.091	1.30	.tau.-Muurolol
64	31.334	4.11	Cyclohexene, 4-pentyl-1-(4-propylcyclohexyl)-
65	31.570	0.54	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha., 4-trimethyl-3-(1-methylethenyl)-, [1R-(1.alpha., 3.alpha.,4.beta.)]-
66	32.074	0.19	3, 7-Cyclodecadien-1-one, 3, 7-dimethyl-10-(1-methylethylidene)-, (E,E)-
67	32.226	0.12	Curlone
68	33.692	20.47	2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-
69	33.895	0.61	(-)-Globulol
70	34.087	0.18	2-((2R,4aR,8aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6, 8a-octahydronaphthalen-2-yl)prop-2-en-1-ol
71	34.291	0.72	Isospathulenol
72	34.442	0.79	Isospathulenol
73	34.700	0.41	3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-
74	34.854	0.18	11-Hydroxy-11-methyl-tricyclo[4.3.1.1(2,5)] undecan-10-one
75	34.949	0.05	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo [4.1.0]hept-1-yl)-ethyl]-vinyl ester
76	35.080	0.15	Ambrial
77	36.066	0.47	9,19-Cyclolanostan-3-ol, acetate, (3.beta.)-
78	36.637	0.05	geranyl-.alpha.-terpinene
79	38.172	0.06	Longifolenaldehyde
80	39.511	0.05	(E)-15,16-Dinorlabda-8(17),11-dien-13-one
81	39.639	0.15	Trachylobane
82	40.171	0.04	cis-Thujopsene
83	40.586	0.12	geranyl-.alpha.-terpinene
84	41.861	0.03	Squalene
85	42.912	0.14	Coronarin E
86	43.093	0.05	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-
87	43.993	1.14	Thunbergol

Discussion

The results of antibacterial activity shows that all ethanolic extracts from rhizomes, stems, leaves and fruits of *Z. rubens* could inhibit both *L. casei* TISTR 390 and *S. mutans* ATCC 25175 at different levels. The present study demonstrates the highest antibacterial activity of stem extract against cariogenic bacteria. However, all aqueous extracts could not inhibit any bacterial strains. The difference in effects between aqueous and ethanolic extracts of the plant might be due to the polarity of solvent extraction that generated various antimicrobial components. Alcohol might solubilize the wider range of compounds in medicinal plants than water. Alcohol is mostly used for extraction of various polar compounds but a certain group of nonpolar compounds was also soluble (Tiwari et al., 2011). The antibacterial activity of *Zingiber* spp. extracts could be expected to be the compounds like flavonoids and volatile oils which dissolved in organic solvents, so the ethanolic extract could have a greater antibacterial activity. Additionally, the essential oil from *Z. rubens* rhizome also inhibited against both bacterial pathogens with inhibition zones of 8.17 and 8.83 mm. According to the GC/MS analysis in this study, zurumbone (20.47%) was found to be the major component of *Z. rubens* oil. This finding is different from the previous study that reported that the major components of *Z. rubens* root oil collected from Vietnam using water distillation were (Z)-citral (30.1%), camphene (9.7%), β-phellandrene (7.5%), 1,8-cineole (7.0%) and zingiberene (5.3%) (Dai et al., 2013). Nonetheless, the composition of the essential oil depended on parts used, geographical distribution and different stages of plant species (Chamorro et al., 2012; Johnson et al., 2004). Zurumbone was previously known for its antimicrobial activity. This compound at concentration between 0.13 - 13 mg/ml, isolated from *Z. zerumbet* could inhibit *Salmonella cholerasuis* by agar disc diffusion method (Abdul et al., 2008). Likewise, the zurumbone derived from *Z. zerumbet* showed MIC of 250 μg/ml MBC of 500 μg/ml against *S. mutans* ATCC 35668 (da Silva et al., 2018). The cytotoxicity assay is one of the reference materials for evaluating the safety screening of bioactive compounds. The current study shows that the ethanolic extracts (30.32-377.35 μg/ml) gives a higher cytotoxicity effect on Vero cells than aqueous extracts (CC_{50}>1,000 μg/ml). Also, the essential oil from rhizomes is not toxic to *in vitro* cells with CC_{50} of 2.5 μg/ml. According to the US National Cancer

Institute, a crude plant extract with CC_{50} less than 20 µg/ml is regarded as having cytotoxicity (Boik, 2001). Therefore, it is clear that the plant extracts and essential oil of *Z. rubens* has no cytotoxicity against normal mammalian cells. However, the *in vitro* cytotoxicity testing was a close system and direct exposure of the cells to bioactive molecules might lead to a high cytotoxicity (Di Nunzio et al., 2017). Furthermore, the chemical kinetics such as absorption, distribution and excretion of the compounds might affect cytotoxic properties of an *in vivo* study (Freshney, 2000). Therefore, the cytotoxicity should also be investigated *in vivo* in the animal models to confirm the effect of extracts in further studies.

Conclusion

In summary, this study suggests that the ethanolic extract and the essential oil of *Z. rubens* shows a promising antibacterial activities against cariogenic species and has a low cytotoxicity. The result suggests that *Z. rubens* might be responsible for the prevention of dental caries.

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Morphological Gill Abnormalities in the Larvae of Hydropsychidae (Trichoptera: Insecta) for Assessment of Water Quality Variables

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Abstract

The aim of this study was carried out to determine the morphological gill abnormalities in hydropsychid larvae in correlation with water quality in the three streams. Samples were collected in April, August and December 2015. In total, 841 individuals were caught belonging to 10 species. Gill abnormalities consisted of dark spots on the gill tufts and reduced numbers of gill filaments. The proportion of individuals with at least some abnormality (HAI), and the average number of abnormal gill tufts for all individuals (HYI) in the Huai Pakkok stream was higher than in the Huai Kayeng stream and the Huai U-Long stream. Physicochemical water quality was slightly differed in each sampling sites. The correlation between normal and abnormal gill morphology and physicochemical water quality parameters were analyzed. The normal and abnormal of *Potamyia phaidra* were correlated with conductivity, total dissolved solids, and alkalinity ($p<0.05$), whereas the *Cheumatopsyche lucida* were correlated with sulfate ($p<0.05$). The normal and abnormal of *Pseudoleptonema quinquefasciatum* and the normal of *Hydropsyche brontes* were correlated orthophosphate ($p<0.05$).

Introduction

The order Trichoptera consists of caddisflies, a group of insects that spend their larval phases in aquatic environments. High species diversity, various ecological and behavioural specializations and very strict environmental requirements, particularly along the longitudinal continuum, make caddisfly larvae excellent study organisms for environmental gradient studies (Morse, 2011; Holzenthal et al., 2007).

The gills of an aquatic macroinvertebrate are one of the most impacted structures on the body of the organism when the environment in which it lives is altered. They are especially sensitive due to their large surface area and their ability to accumulate compounds and gases (Laporte et al., 2002). One of the most important biological indicator taxa for water quality is Trichoptera (caddisflies). This group of insects is ideal because it is high in biodiversity, inhabits many ecological niches, and is abundant (Dohet, 2002; Resh, 1993; Rosenberg & Resh, 1993; Wiggins, 1996). However, many caddisfly species are known for their intolerance of habitat sedimentation and organic pollution (Barbour et al., 1999), and so their numbers may strongly diminish

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downstream of habitat disturbances (Berlin & Thiele, 2002). In contrast, some species are more tolerant of organic pollution and may increase in abundance of such downstream disturbances (Barbour et al., 1999).

The caddisflies family Hydropsychidae have been increasingly utilized in biomonitoring and impact assessment of pollutants in rivers for several reasons (Vuori, 1994; 1995; Vuori & Kukkonen, 1996). First, hydropsychid larvae are widely distributed and abundant in many types of running waters. Second, they respond to variations in water quality and their autecology is well known enough for the impact of pollutants to be distinguished. Third, due to their robust body, hydropsychid larvae are easily handled and observed for morphological abnormalities. Fourth, the abnormalities in the hydropsychid tracheal gills, the ion-regulatory organ and the anal papillae can be attributed to a disruption of the respiratory and ion regulation functions. Fifth, the relatively large size facilitates sampling and analysis of the concentrations of chemicals in the larvae. Finally, the hydropsychid larvae as facultative filter feeders are more exposed to pollutants in seston, flowing water and the organic matter accumulated in riffle microhabitats. The gills of hydropsychid larvae are one of the most impacted structures on the body of the organism when the environment is altered. They are particularly sensitive due to their large surface area, which increase the accumulation of compounds and gases (Skinner & Bennett, 2007). Direct effluent discharges and agricultural runoff water mostly contain complex mixtures of contaminants that may produce new compounds due to breakdown and transformation processes and hence contribute to the complexity of the total toxin burden. By the employment of chemical and physical measurements only, the synergistic effect of pollution on its biotic community may not be fully and easily assessed (Resh & Jackson, 1993). In general, biological indicators provide a potential for direct observation of the overall effect of environmental contaminants by virtue of their role in aquatic ecosystems (Warwick, 1988). The purpose of this study was to investigate individual gill morphology alterations in hydropsychid larvae and to consider possible impacts of water quality parameters (e.g., dissolved oxygen (DO)), pH, water temperature, conductivity, total dissolved solids (TDS), sulfate and nutrients on gills morphological structure.

Materials and methods

1. Study area

We used the samples from streams in the Mae Klong watershed, which is the most important watershed in the western part of Thailand. The upstream area consists of the Khwae Noi and Khwae Yai rivers, namely, that run into the Khao Leam and Srinagarind Dam located in the upper region of Mae Klong watershed. The rivers are jointed downstream in Kanchanaburi Province, flowing through Ratchaburi and Samutsongkram provinces finally flowing into the Gulf of Thailand. The sampling sites were three streams on the upper reach of the Khwae Noi River, Kanchanaburi Province, Thailand, upstream from Khao Leam Dam: Huai U-Long (UL1), Huai Pakkok (PK1, PK2), and Huai Kayeng (KY1, KY2, KY3) (Fig. 1). These streams are major sources for household and irrigation water supply in Thong Pha Poom District, Kanchanaburi Province.

2. Physicochemical water quality parameters

Selected physicochemical water quality parameters were recorded directly at the sampling site and included pH (measured by a pH-meter Waterproof Model Testr30), water temperature (measured by a hand-held thermometer), and dissolved oxygen (DO, measured by a HACH® Model sensION 6 DO meter), total dissolved solid (TDS) and electrical conductivity (EC) (measured by a EURECH CyberScan CON110 conductivity/TDS meter). Water samples from each collecting period were stored in polyethylene bottles (500 mL). Ammonia nitrogen (NH_3-N), sulfate (SO_4^{2-}), nitrate-nitrogen (NO_3^--N), orthophosphate (PO_4^{3-}), and turbidity were determined in accordance with standard procedures (APHA., 1992). Alkalinity was measured by titration (APHA., 1992).

3. Sample collection and identification

The hydropsychid larvae were collected from mid- and downstream riffle areas of the three tributaries stream of the Mae Klong watershed in April, August and December 2015. Larvae were handpicked from stones, stone crevices, gravel, woody debris and other stable substrates. Sampling time of the larvae was restricted to one hour for standardization. The specimens were then preserved in 80% EtOH and brought to the laboratory where they were sorted to morphospecies using characters in the key for Thai caddisfly larvae as described in Prommi (2007). At the same sites where larvae and pupae were collected, adults were collected at the stream margin using black light traps operated from after sunset until morning.

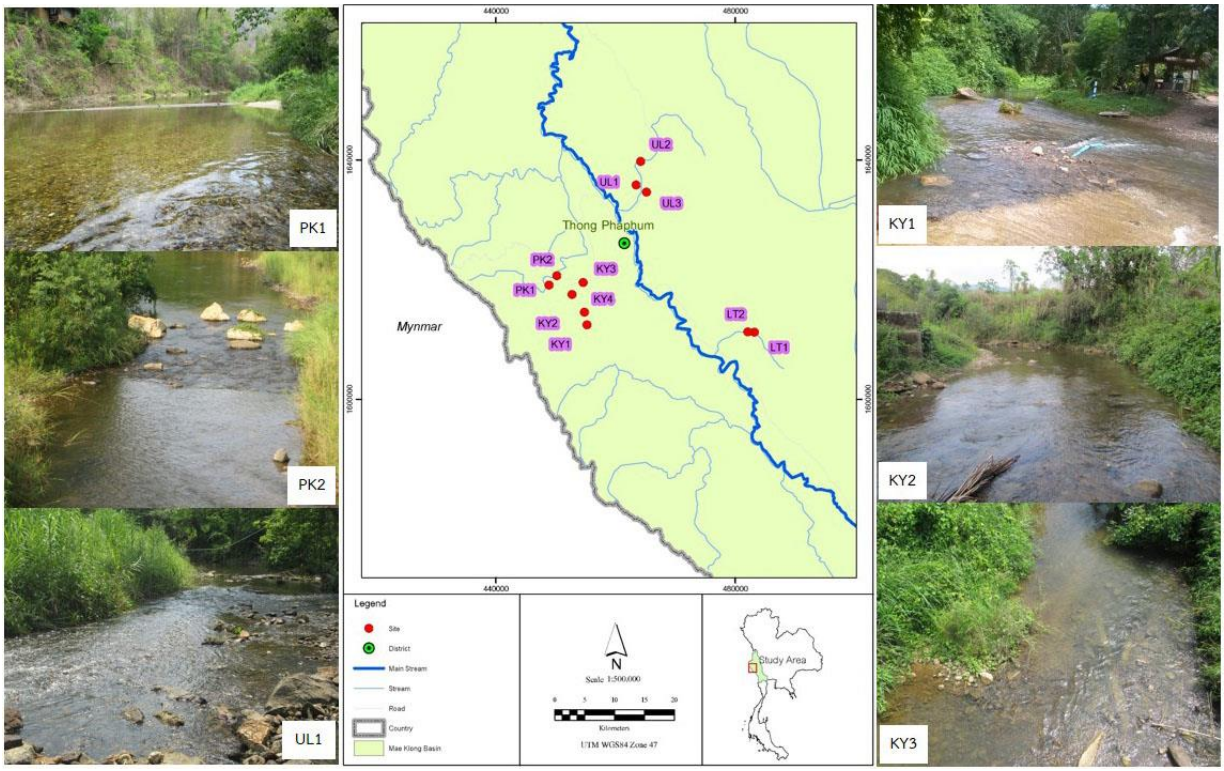

Fig. 1 Map of the Mae Klong watershed showing the sampling sites in three streams, Huai Kayeng (KY1, KY2 and KY3), Huai Pakkok (PK1, PK2) and Huai U-Long (UL1).

The immature forms were associated with adults using the metamorphotype method, which relies on the collection of a pharate male in its pupal exuviae and with shed larval sclerites within the pupal case or shelter (Milne, 1938; Wiggins, 1996).

4. Analyses

Structural changes in the hydropsychids gills were studied under a stereomicroscope and quantified using an ocular micrometer. Small and light pigmentation spots were considered part of natural variation and so were not categorized as a morphologically abnormal. Two biomarkers were evaluated: (1) Hydropsychid abnormality incidence (HAI), referring to the proportion of individuals with at least some abnormalities, and (2) Hydropsychid gill abnormality indice (HYI), referring to the average number of abnormal gill tufts for all individuals (Vuori, 1994; Vuori & Kukkonen, 1996). Correlations between all variables for water quality, and hydropsychid larvae were tested by Pearson product-moment correlation coefficient using SPSS v. 13.0 (http://www.spss.com/).

Results and discussion

1. Physicochemical parameters of water quality

Mean values of selected physicochemical parameters of water quality at n the three tributaries stream of Mae Klong watershed during this study are presented in Table 1.

The mean value of water temperature ranged from 25.22±2.28 (UL1) to 28.86±3.36°C (PK2). Temperatures were relatively lower during the wet season than during the dry season. The minimum (25.0ºC) and maximum temperatures (35.5ºC) were normal for tropical waters and were required for the normal growth of aquatic organisms. At the upstream site, the riparian areas were covered by vegetation that shaded the water and may have provided lower water temperatures. This is similar to reports of Hauer & Hill (1996) that a smaller watershed stream has lower temperature because the riparian trees help protect the water from heat.

The mean value of pH ranged from 8.26±0.10 (UL1) to 8.69±0.36 (KY1). The accumulation of free carbon

dioxide due to reduced photosynthesis by phytoplankton and rooted macrophytes causes lower pH values in water while intense photosynthesis reduces free carbon dioxide content and results in higher pH values (Egborge, 1994; Gupta & Gupta 2006). The pH was decreased in the rainy season. This may be attributed to the increased organic matter washed into the stream by surface runoff during the wet season that tends to reduce dissolved oxygen through organic decomposition, reducing pH.

The mean value of dissolved oxygen ranged from 4.61±2.15 mg/L (KY2) to 5.90±1.74 mg/L (KY1). Sources of dissolved oxygen in aquatic environments include the atmosphere and photosynthesis and its concentration depends on its solubility (which decreases with increasing water temperature), while dissolved oxygen is reduced by respiration of submerged plants and animals and aerobic bacteria, including their metabolic activity in decomposing dead organic matter (Gupta & Gupta 2006). The water current at KY1 was high, resulting in a turbulent flow which increases oxygen in the water continually (Boulton & Brock 1999).

Mean electrical conductivity values ranged from 68.98±43.58 µS/cm (KY1) to 243.41±103.96 µS/cm (KY3). The mean total dissolved solids values ranged from 35.87±19.92 mg/L (KY1) to 121.79±51.91 mg/L (KY3). The total dissolved solids and electrical conductivity were highest in KY3 and lowest in KY1. The general trend in this study was that electrical conductivity tended to decrease in the dry season compared with the wet season. Increases in electrical conductivity could result from low precipitation, higher atmospheric temperature resulting in higher evapotranspiration rates and higher total ionic concentration, and saline intrusions from underground sources. It could also be due to a high rate of decomposition and mineralization by microbes and by nutrient regeneration from bottom sediments (Egborge, 1994). Also, electrical conductivity and total dissolved solids could be higher due to contamination of water from agricultural activities and industrial effluent that degrade water quality (Lenat & Crawford, 1994).

The mean turbidity values ranged from 4.11±4.00 NTU (PK1) to 27.56±26.94 NTU (UL1). The higher turbidity was recorded during the wet season and may have been due to heavy rainfall. This increase in suspended solids impeded light thereby increasing turbidity. The adverse effects of turbidity on freshwater include decreased penetration of light which then reduces primary and secondary production, increased adsorption of nutrient molecules to suspended materials which makes the nutrients unavailable for plankton production, decreased oxygen concentration, and clogged filter-feeding apparatus and digestive organs of planktonic organisms, which may adversely affect the production of larvae (Gupta & Gupta, 2006).

Mean alkalinity values ranged from 11.87±5.89 mg/L (PK1) to 35.47±9.20 mg/L (KY3). Water bodies in the tropics usually show wide fluctuations of total alkalinity, the values depending on the location, season, plankton population and nature of bottom deposition. Highly productive waters have alkalinity values above 100 ppm and for freshwater aquaculture the values should be between 40-200 ppm. Alkalinity values above 300 ppm have been reported to affect the spawning and hatching of freshwater fish adversely (Gupta & Gupta, 2006).

The mean dissolved nutrients, NH_3-N, PO_4^{3-}, and NO_3^--N concentrations varied from 0.18±0.07 mg/L (PK2) to 0.31±0.12 mg/L (KY1), 0.32±0.16 mg/L (PK1) to 0.93±0.57 mg/L (KY1), and 0.92±0.16 mg/L (PK2) to 1.54±0.45 mg/L (KY3), respectively. The mean concentration value of SO_4^{2-} ranged from 1.00±0.25 mg/L (PK1) to 5.00±0.25 mg/L (PK2). Nitrates are the most

Table 1 Physicochemical parameters at sampling sites on Mae Klong watershed, showing mean and standard deviation for each site in April, August and December 2015

Parameter/site	PK1	PK2	KY1	KY2	KY3	UL1
WT (ºC)	26.18 ± 2.44	28.86 ± 3.36	28.59 ± 5.65	28.07 ± 3.91	25.95 ± 1.20	25.22 ± 2.28
pH	8.50 ± 0.09	8.40 ± 0.15	8.69 ± 0.36	8.55 ± 0.11	8.45 ± 0.04	8.26 ± 0.10
DO (mg/L)	5.07 ± 2.21	5.23 ± 3.64	5.90 ± 1.74	4.61 ± 2.15	5.79 ± 1.28	5.74 ± 4.29
EC (µS/cm)	72.39 ± 20.46	114.08 ± 40.19	68.98 ± 43.58	238.43 ± 82.47	243.41 ± 103.96	239.22 ± 39.46
TDS (mg/L)	38.36 ± 7.78	55.28 ± 20.75	35.87 ± 19.92	113.33 ± 44.79	121.79 ± 51.91	119.94 ± 19.41
Turbidity (NTU)	4.11 ± 4.00	7.44 ± 4.44	6.22 ± 4.60	11.33 ± 10.37	6.33 ± 3.76	27.56 ± 26.94
Alkalinity (mg/L)	11.87 ± 5.89	15.13 ± 5.19	12.13 ± 1.31	30.67 ± 8.61	35.47 ± 9.20	32.67 ± 8.61
NH_3-N (mg/L)	0.26 ± 0.18	0.18 ± 0.07	0.31 ± 0.12	0.26 ± 0.06	0.26 ± 0.05	0.27 ± 0.21
PO_4^{3-} (mg/L)	0.32 ± 0.16	0.51 ± 0.16	0.93 ± 0.57	0.40 ± 0.15	0.39 ± 0.16	0.83 ± 0.80
NO_3^--N (mg/L)	1.46 ± 0.46	0.92 ± 0.16	1.37 ± 0.63	1.21 ± 0.43	1.54 ± 0.45	1.24 ± 0.18
SO_4^{2-} (mg/L)	1.00 ± 0.25	5.00 ± 0.25	1.00 ± 0.41	4.00 ± 2.25	5.00 ± 3.25	2.00 ± 1.25

oxidized forms of nitrogen and the end product of aerobic decomposition of organic nitrogenous matter. Natural waters in their unpolluted state contain only minute quantities of nitrates. The highest nitrate values occurring during the monsoon/post monsoon season may be primarily due to organic materials being received from the catchment area during rainfall (Das et al., 1997). The increasing levels of nitrates are due to freshwater inflow, litter fall decomposition and terrestrial run-off during the monsoon/post monsoon season (Karuppasamy & Perumal, 2000). Another possible source of nitrate recruitment is through oxidation of ammonia from nitrogen to nitrite formation (Rajasegar, 2003). The low values during the summer/pre-monsoon period may be due to utilization of nitrates by phytoplankton as evidenced by high photosynthetic activity. In addition, nitrates may be obtained from natural and human activities, both from household wastes and from fertilizers used in agriculture, consistent with observations by Omernik (1977) who indicated that the levels of nutrients in streams were positively correlated with percentage of land in agriculture. Furthermore, although orthophosphate is usually a minor compound in nature (Jarvie et al., 2002), its higher values in stream water at our sampling sites was from agricultural fertilizers which are leached into water when it rains.

2. Biodiversity of Hydropsychidae

Eighteen species of adult hydropsychids were caught by light trapping in the three tributaries of Mae Klong watershed during this study. The most species rich genera were *Cheumatopsyche* (6 species), followed by *Macrostemum* (3 species), *Potamyia* (3 species), *Hydropsyche* (2 species), and *Amphipsyche, Diplectrona, Polymorphanisus,* and *Pseudoleptonema* (each 1 species) (Table 2).

Totally, 844 individual of hydropsychid larvae were collected in the three tributaries of Mae Klong watershed during this study (Table 3). Of these, 9 larvae were associated with identifiable adults of Hydropsychidae: *Cheumatopsyche copia, Cheumatopsyche lucida, Hydropsyche brontes, Hydropsyche camillus, Potamyia phaidra, Pseudoleptonema quinquefasciatum, Polymorphanisus astictus, Macrostemum floridum* and *Amphipsyche gratiosa* (Table 3). The most abundance of hydropsychids collected was found in KY1 (272 individuals) which was abundant than in PK2, PK3, KY1, KY2 and UL1 (224, 218, 92, 27 and 8 individuals, respectively).

Gills were considered impaired if they exhibited heavy darkening, malformation, and/or reduction of single gill tufts (Fig. 2). Darkening of the gills appeared to start either at the basal or distal ends. We considered minor darkening (often single pigmented spots) not to represent a morphological abnormality because potentially this might be induced by natural causes (Fig. 3). The HAI in Huai Pakkok (PK1, PK2) was 71.56% and 50%, in Huai Kayeng (KY1, KY2 and KY3) was 26.47%, 16.30% and 6.67% and in Huai U-Long was 12.5%. The HYI was highest value in Huai Pakkok (9.34%, 5.06%), followed by Huai Kayeng (4.83%, 1.53%, 0.46%) and Huai U-Long (0.8%) (Table 3). In this study, *Potamyia phaidra* larvae were most abundant in all streams and the gills of this species were most impaired.

Table 2 Checklist of adult Hydropsychidae at three streams, six sampling stations of the Mae Klong Watershed in April, August and December 2015

Species	Station
Amphipsyche gratiosa Navas 1922	KY1, KY2, PY2
Cheumatopsyche charites Malicky & Chantaramongkol 1997	PK1, PK2, KY1, KY2, UL1
Cheumatopsyche cocles Malicky & Chantaramongkol 1997	PK1
Cheumatopsyche lucida Ulmer 1907	PK1, UL1
Cheumatopsyche chrysothemis Malicky & Chantaramongkol 1997	KY2
Cheumatopsyche dubitans Moesly 1942	PK1, PK2
Cheumatopsyche globosa Ulmer 1910	PK1, KY2, UL1
Diplectrona gombak Olah 1993	KY2
Hydropsyche brontes Malicky & Chantaramongkol 2000	PY1, KY1
Hydropsyche camillus Malicky & Chantaramongkol 2000	KY1
Macrostemum dohrni Ulmer 1905	PK1
Macrostemum floridum Navas 1929	PK2, KY2, UL1
Macrostemum midas Malicky & Chantaramongkol 1998	PK1, PK2, KY1, KY2
Polymorphanisus astictus Navas 1923	PK1
Potamyia chaos Malicky & Thani 2000	PK1
Potamyia flavata Banks 1934	PK1
Potamyia phaidra Malicky & Chantaramongkol 1997	PK1, PK2, KY1, KY2
Pseudoleptonema quinquefasciatum Martynov 1935	PK1, PK2, KY1, KY2, UL1

3. Correlation between physicochemical parameters and hydropsychid larvae

The correlation between physicochemical water quality parameters and hydropsychid larvae were analyzed. The Hydropsychidae *Potamyia phaidra* were negatively correlated with electrical conductivity, total dissolved solids and alkalinity ($p<0.05$), whereas *Cheumatopsyche lucida* were negatively correlated with sulfate ($p<0.05$). The *Pseu. quinquefasciatum* and *Hydropsyche brontes* were negatively correlated with

Table 3 Total number of hydropsychid larvae collected in three streams, six sampling stations of the Mae Klong Watershed in April, August and December 2015

Site	Species	Total	Individual		Total gills	Gill abnormal	HAI (%)	HYI (%)
			Normal	Abnormal				
PK1	*Pseu. quinguefasciatum*	49	36	13	2796	27	26.53	0.96
	Hydropsyche brontes	21	7	14	630	38	66.66	6.03
	Hydropsyche camillus	1	0	1	30	1	100	3.33
	Polymorphanisus astictus	1	1	0	60	0	0	0
	Potamyia phaidra	148	66	82	4440	332	55.4	7.47
	Cheumatopsyche lucida	3	2	1	105	2	33.33	1.9
	Macrostemum floridum	1	0	1	29	7	100	24.14
PK2	*Potamyia phaidra*	181	38	143	5430	563	79	10.36
	Hydropsyche brontes	6	3	3	180	9	50	5
	Cheumatopsyche lucida	1	1	0	35	0	0	0
	Macrostemum floridum	6	1	5	174	29	83.33	16.66
	Hydropsyche camillus	23	18	5	690	12	21.74	1.74
	*Pseu. quinguefasciatu*m	1	1	0	57	0	0	0
KY1	*Cheumatopsyche cop*ia	24	12	12	840	23	50	2.74
	Cheumatopsyche lucida	17	16	1	595	1	5.88	0.17
	Hydropsyche brontes	38	31	7	1140	15	18.42	1.32
	Macrostemum floridum	192	138	54	5568	356	28.12	6.39
	Potamyia phaidra	1	1	0	30	0	0	0
KY2	*Potamyia phaidra*	13	10	3	390	6	7.69	1.54
	Hydropsyche brontes	56	48	8	1680	19	14.29	1.13
	Pseu. quinguefasciatum	6	6	0	342	0	0	0
	Amphipsyche gratiosa	17	17	0	48	0	0	0
KY3	*Potamyia phaidra*	22	21	1	660	1	4.54	0.15
	Amphipsyche gratiosa	3	3	0	48	0	0	0
	Pseu. quinguefasciatum	2	2	0	114	0	0	0
UL1	*Pseu. quinguefasciatum*	5	5	0	285	0	0	0
	Hydropsyche camillus	3	2	1	90	3	33.33	3.33

Fig. 2 Hydropsychidae larvae, *Amphipsyche gratiosa* (left) and *Pseudoleptonema quinquefasciatum* (right) showing the impairment of gill morphology.

Fig. 3 Hydropsychidae larvae, *Cheumatopsyche lucida* (left) and *Macrostemum floridum* (right) species showing the normal gill morphology.

orthophosphate ($p<0.05$) (Table 4). The results suggest that physicochemical attributes such as electrical conductivity, total dissolved solids, alkalinity, orthophosphate, and sulfate significantly contributed to the impairment of hydropsychid larvae gills in the Mae Klong tributaries.

The biological method of water quality analysis has certain advantage over the physicochemical analysis. In particular, developing countries could benefit more from this type of analysis rather than using the costly physicochemical one. Among the various biological methods, incidence of deformities among benthic macroinvertebrates is getting due attention to assess the health of aquatic ecosystems. However, deformity could also result from factors other than pollution. That is why care has to be taken when associating deformity with pollution or environmental stress (Beneberu & Mengistou, 2014).

The previous study in gill morphology deformities in Hydropsychidae larvae have been reported by Prommi & Thamsenanupap (2013). This paper presents a quantitative study on gill abnormalities in population of hydropsychid larvae in stream from Mae Klong watershed, and all showed heavily darkened, malformed and/or reduced single gill tufts. The gill pigmentation was obvious, starting from either basal or distal ends. Completely total dissolved solids, alkalinity and orthophosphate concentration were negatively correlated with gill abnormalities, whereas the pH and

Table 4 Pearson's correlation coefficients between water quality variables and hydropsychid larvae impairment

Species	EC	TDS	Alkalinity	SO_4^{2-}	PO_4^{3-}
Potamyia phaidra (normal gills)	-0.916*	-0.907*	-0.855*		
Potamyia phaidra (gills abnormalities)	-0.854*	-0.845*	-0.836*		
Cheumatopsyche lucida (gills abnormalities)				-0.816*	
Pseu. quinquefasciatum (normal gills)					0.905*
Pseu. quinquefasciatum (gills abnormalities)					0.845*
Hydropsyche brontes (normal gills)					0.845*

Remarks: * : Significant at the 0.05 level, negative (-).
* : Significant at the 0.05 level, positive (+).

Cu concentration parameters were positive correlated.

The capacity of water to accept hydrogen ions is called alkalinity and is important in the chemistry and biology of natural waters. Alkalinity serves as a pH buffer and reservoir for inorganic carbon, thus helping to determine the ability of water to support algal growth and other aquatic life. Alkalinity can be used as a measure of water fertility. The distinction between elevated pH and high alkalinity is important. The pH is an intensity factor whereas alkalinity is a capacity factor. The greater alkalinity and hardness in stream water most likely accounted for the reduced Cu toxicity observed in stream. Gauss et al. (1985) compared effects of Cu on chironomids in hard and soft water. They reported 96-h LC_{50} values of 17 µg Cu/Lin soft (43 mg/L $CaCO_3$) water and 98 µg Cu/L in hard (172 mg/L $CaCO_3$) water. In contrast from this study, the alkalinity in Huai Pakkok (PK1 and PK2) was the lowest, whereas Cu was the highest. Hardness was not measured in our streams and the influence of this parameter on observed Cu toxicity is unknown.

Elevated levels of total dissolved solids (TDS) have been suggested as stressors to aquatic life in Central Appalachian streams influenced by coal mining (Bodkin et al., 2007; Pond et al., 2008). In coalfield streams, TDS is most often dominated by the dissolved ions SO_4^{2-} and HCO_3^-, with elevated concentrations (relative to reference) of Ca^{2+}, Mg^{2+}, Na^+, K^+, and Cl^- also common (Pond et al., 2008; Mount et al., 1997). At present here, *Cheumatopsyche lucida* were negatively correlated with sulfate. This suggests that while the number of taxa present may increase with increasing TDS, abundance of individuals within the remaining species, and perhaps overall species abundance, may remain less affected, at least within the range of TDS.

Conclusion

The present study clearly indicated that the stream from Mae Klong watershed is experiencing pollution from domestic activities established near its vicinity. The domestic pollution load in the river can be witnessed by deformed structures manifested on hydropsychid gill morphology. Various forms of deformities were noted with highest incidence occurring on heavy darkening, malformation, and/or reduction of single gill tufts. Therefore, it is possible to conclude that morphological deformities among Hydropsychid larvae such as *Potamyia phaidra, Cheumatopsyche lucida, Pseu. quinquefasciatum* and *Hydropsyche brontes* can be a potential tool to assess the health of an aquatic ecosystem. However, future studies should focus not only on the field data but also on laboratory-based experiments on live hydropsychid larvae. With this approach, it is possible to explain the major environmental variables that can cause the highest rate of deformity among hydropsychids.

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Property Changes of the Traditional Beef Curry Product *'Gulai-Besar'* Packed in Retort Pouch during Storaging

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Abstract

The main objective of this study was to figure the changes of physicochemical as well as the sensory properties of the traditional beef curry product *'Gulai-Basar'* packed in retort pouch during storage up to 9 months. *Gulai-Besar* was prepared according to the local recipe. It was packed in retort pouch (250 g) and processed in a retort at 116°C for 26 minutes and the F_0 Value of 5.77. The retort processed *Gulai-Besar* was analyzed for microbiological, sensory and chemical characteristics under refrigerated (4±2°C), ambient temperature (30±2°C), and accelerated temperature (55±2°C) for 9 months. All the processed samples showed an increasing trend of peroxide value (PV), free fatty acid (FFA), ash and sensory attributes while a decreasing trend of pH, protein and moisture content were showed. The fat slightly increased during storage time in all conditions. However, mesophile aerobes and anaerobes were absent and the samples were safe with acceptable sensory attributes entire storage period. The shelf life study indicated that the products were well acceptable up to 9 months based on the assessment of physicochemical, microbiological and sensory attributes. This implies that it is feasible to produce *Gulai-Besar* to commercial scale.

Introduction

Traditional foods play a crucial role in local identity, consumer behavior, the transfer of cultural heritage for future generations, and also the interaction of the interaction of this heritage with the remainder of the globe (Albayrak & Gunes, 2010). In the south of Thailand, *Gulai-Besar* could be a classic ancient beef curry. It mostly consists of large pieces of meat with coconut milk and varied spices. Quite often, this local cuisine is tied to some form of religious occasions such as Eid festival, Ramadan, and also the bridal ceremony. *Gulai-Besar* is commonly consumed among the Muslim households of Thailand and other parts of the Nusantara region (Southeast Asia).

The ready to eat (RTE) meal market has grown significantly in recent years because of an increasing demand for convenience food (Van der Horst et al., 2010). Retort pouch processing technology has been widely recognized as one of alternative to metal containers

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because of their lighter, more appealing, and convenient end use (Al-Baali & Farid, 2006). It is a thermal process that imparts increased shelf life with good retention of nutrients and sensory parameters.

People are buying different type of food as lifestyle changes; work hours increase and more women work outside the home. Speed and convenience are increasing priorities for busy consumers short on time work, driving sales of instant foods. Thus, food companies need to keep ahead of the trends and competition because the consumer would prefer their traditional food on daily basis.

The objective of this study was to investigate a method for thermal processing of *Gulai-Besar*, a traditional Muslim dish packed in pouches and process in retort. The task was to evaluate the product in regard to physicochemical properties, microbiological status and sensory quality in different storage conditions.

Materials and methods

1. Preparation of *Gulai-besar*

Fresh deboned beef was collected from private beef farm, slaughtered by halal method. Beef was trimmed-off lean and cut into a piece of 1x2 inches (W/L) using sterilized meat cutting knife. The pieces were roasted at 80±2°C for 10 minutes and dried using a fan for 30 minutes. The beef was packed in highdensity polyethylene (HDPE) bags and stored at -20±2°C till further use. The formulation for the *Gulai-Besar* was standardized from the popular restaurant of Betong named "Hantana restaurant", Thailand. The ingredients were purchased from local markets, presented in Table 1 and the making process is illustrated in Fig. 1.

Table 1 Recipe of the curry sauce for *Gulai-Besar*

Ingredients	Percentage, %	Ingredients	Percentage, %
Beef	45.00	Curry Powder	2.20
Coconut Milk	24.75	Lime Juice	2.00
Shallot	8.25	Roasted Coconut	1.65
Garlic	3.85	Ginger	1.65
Palm Sugar	3.85	Shrimp Paste (Shrimp, Salt)	0.55
White Sugar	3.30	Dry Chili	0.55
Coconut Sugar	2.20	Spices	0.20

2. Retort pouch processing

Retortable pouches (140x180 mm) made with four layers (PET12/ NY15/Al9/CPP80 Microns) were purchased from PAYA BELL Corporation Co., Ltd., Pathum Thani, Thailand. The beef (80 g) were filled in

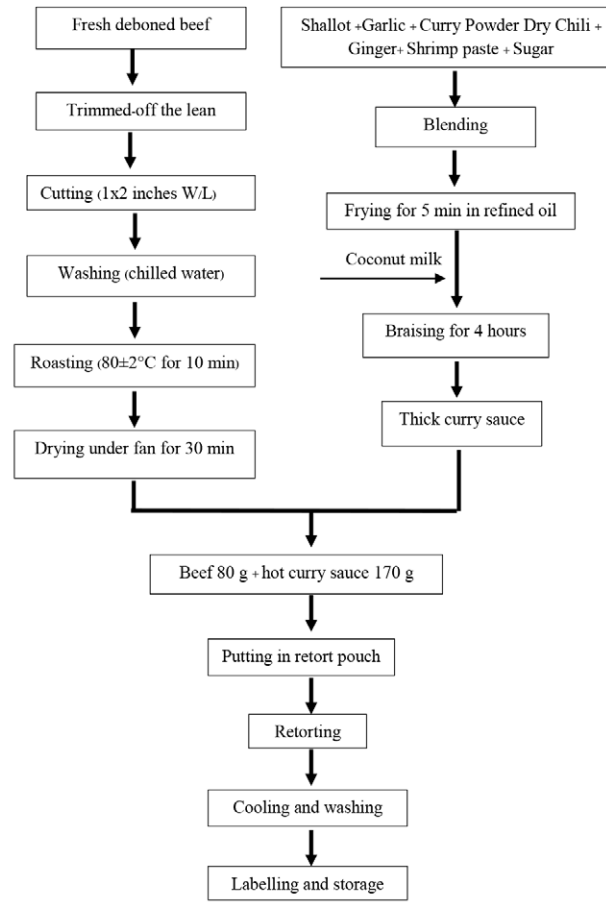

Fig. 1 The process of Gulai-besar in retort pouch.

pouches with 170 g hot curry sauce, maintaining a pack weight of about 250 g. Residual air was removed manually and sealed by impulse sealing machine. All 10 temperature measurement lines are placed between multiple packaging points in the machine to find the coldest heat point. Sealed pouches were subjected to thermal processing by using steam-air moisture heating (model PP500, capacity 500 liters, size 1690x2400x2030 mm) and water-air mixture was used while cooling. The product core temperature was recorded every 60 seconds using the Programmable Logical Control Device (E Val Pro System, Ellab, Denmark). The factor of heating (I_h), slope of the heating curve (f_h), and time for sterilization (U) were calculated. The heat penetration data were plotted on semi-log paper with temperature deficit ($RT-T_c$) on the log scale against timing. The parameters f_h/U, process time, F_0 value and Total process time were

calculated by the mathematic method (Patashnik' method lethal rate summation). Retort pouch processing of *Gulai-Besar* was done at Halal Food Science Center (HFSC), department of Food Science and Technology, Prince of Songkla University, Pattani, Thailand.

3. Storage analysis

Gulai-Besar was stored under different temperatures, i.e. refrigerated temperature (4±2°C), ambient temperature (30±2°C), and accelerated temperature (55±2°C). The samples were analyzed to determine the changes occurred in physicochemical properties, microbial analysis and sensory attributes during 9 months storage.

4. Proximate and chemical analysis of *Gulai-Besar*

The moisture content, protein, lipid and ash content were following AOAC method (AOAC., 2000). The carbohydrate content was calculated by subtracting the percentages of moisture, crude protein, crude fat and total ash from 100. Percentage of free fatty acid (FFA) as palmitic with the AOCS Ca 5a–40 standard method (Firestone, 2004) and peroxide value (PV) were analyzed according the AOAC (2000). The pH was measured by using a digital pH meter (Cyber Scan pH, 510, Merck).

5. Microbiological analysis

The *Gulai-Besar* was analyzed for its commercial sterility. The sample from each condition were determined for total viable count, anaerobic count, *Staphylococcal*, yeast and mold count, and *Clostridium botulinum* by the method of FDA BAM Online (2001). *E. coli* and coliform were examined by the method of FDA BAM Online (2002).

6. Sensory evaluation

The *Gulai-Besar* was evaluated at the interval of 3 months for quality and acceptability. Trained panelists required to evaluate the color, flavor, taste, texture and overall acceptability of the sample, using 9 points hedonic scale (1=dislike extremely, 9=like extremely).

7. Statistical analysis

The statistical analysis was done by SPSS software package for windows (SPSS Version 20; SPSS Inc., Chicago, USA). Data were analyzed by ANOVA and means were separated using Duncan's multiple range test and statistically significant was determined at 95% confidence level ($p<0.05$). All determination was made in triplicate, and data were expressed as mean with standard deviations.

Results and discussion

1. Effect of retort processing on RTE *Gulai-Besar*

RTE *Gulai-Besar* was prepared based on the culinary style preferred in the locality. The product developed was processed at temperature of 116°C and a reference temperature was used to calculate the process lethality for *C. botulinum*. Thermal processing experiment indicated F_0 value of 5.77 as optimum of achieving commercial sterility of retort process of *Gulai-Besar*. The come-up time (CUT) at the first lethality rate of 0.01 after 26 min of processing when the product core temperature was 87.38°C and retort chamber temperature was 116.63°C. Other parameters of the retort processed are shown in Table 2 and the time-temperature history curves of the products are illustrated in Fig. 2. Our results were similar to previous study in which the F_0 value of Indian traditional type chettinad chicken product was 5.2 (Chandirasekaran & Rajan, 2016).

Table 2 Heat penetration characteristics of *Gulai-Besar* in retort pouch

Parameters	Values
Com up time (CUT)	26
Heating time (HT)	36
Cooling time (CT)	11
Total process time (CUT+HT+CT)	73
F_0	5.77
Heating rate index (f_h), min	26.1
Process time, min	47
Cook Value, min	117.42
F_h/U	1.392

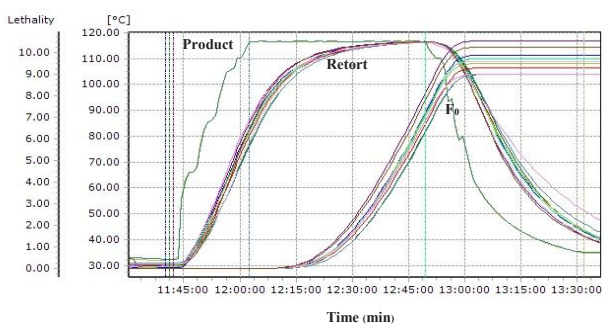

Fig. 2 Heat penetration characteristics of *Gulai-Besar* in retort pouches at the retort temperature of 116 °C.

The process lethality adopted was in agreement within recommendations and findings of other authors. Chanderasekaran and Rajan (2016) found that Indian traditional type chettinad chicken in retort pouches at 121.1°C and corresponding F_0 was sufficient to be able to store the product at 35±2°C for 180 days. Drotz (2012)

recommended a process lethality as low as 6 minutes can be used to achieve a product with better sensory and nutritional qualities.

2. Proximate and chemical analysis of *Gulai-Besar*

Table 3 shows the proximate compositions of the RTE Gulai-Basar under different storage conditions. It was found that the protein and moisture content were decreased while the fat and ash content were increased with increase in storage time. The moisture reduced after processing due to heat penetration of protein (Rajkumar et al., 2010). According to Leander et al. (1980), when meat is cooked, water and soluble protein are expelled from the tissue. The higher value of fat and ash can be attributed to coconut milk and spices added during cooking (Sunooj & Radhakrishna, 2013). The results from measurement of pH are shown in Fig. 3. The pH of the products had declined gradually from 5.89 to 5.26 with increasing period of storage. As expected, these parameters have values allowing growth of proteolytic *C. botulinum* at pH>4.6 and a_w>0.94. The results supported the use of a high temperature treatment for reduction of bacteria spore (Drotz, 2012). A decreasing trend of pH during storage could be contributed by the curry ingredients like dried chili and lime juice. Further, the changes might be cause by the degradation of proteins and liberation of free amino acids (Devadason et al., 2014). A decrease in pH values had also been observed during storage of Fish Peera (Bindu et al., 2010), chettinad goat meat curry (Rajkmar et al., 2010) and chettinad chicken (Rajan et al., 2014) processed in retort pouches.

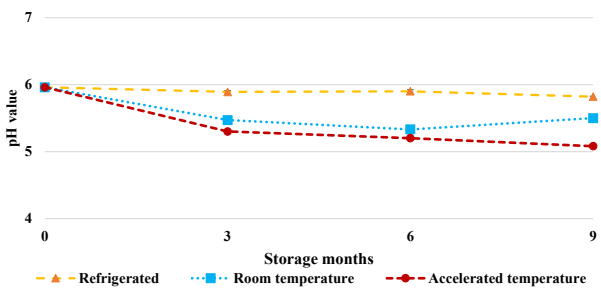

Fig. 3 Change in pH of RTE *Gulai-Besar* during different storage conditions up to 9 months.

The free fatty acid values (FFA) changed significantly during storage period at different temperature. The FFA value in *Gulai-Besar* increased with increased in storage time and the highest value was observed in 9th month of storage up to 5.08±0.10 and 8.14±0.25 % of palmitic acid under ambient temperature (30±2°C) and accelerate temperature (55±2°C) storage, respectively. A significant and steady increase of free fatty acids from 3.40 to 8.14 (% of palmitic acid) was found with increase of storage. Thermal processing at high temperature might have caused an increase in the rate of fat break down into glycerine and free fatty acid resulting more free fatty acids during storage.

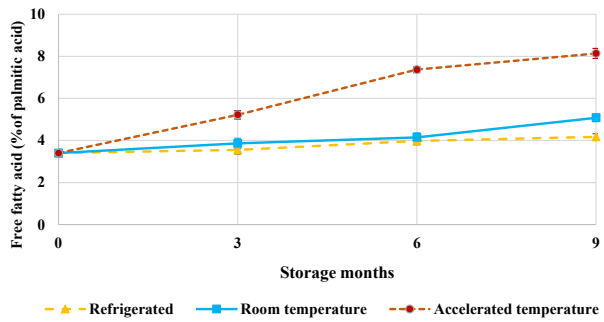

Fig. 4 Change in free fatty acid (FFA) content of RTE *Gulai-Besar* during different storage conditions up to 9 months.

Lipid oxidation in meats might be prepared whereby polyunsaturated greasy corrosive respond with receptive oxygen species, driving to the corruption of lipid and advancement of oxidative rancidity (Amaral et al., 2018). The oxidative degradation of lipid is a major cause of deterioration in the quality of meat and meat products. The peroxide value (PV) of *Gulai-Besar* during storage was slowly increased in range of 0.32-0.42, 0.46-0.68 and 0.54-1.04 mg Eq/kg under refrigerate, ambient and accelerate temperature storage, respectively. The results indicated that the oxidative rancidity development was temperature dependent (Abhishek et al., 2014).

Coconut milk is the main ingredient in *Gulai-Besar* and it is susceptible to chemical change, especially change of the PV and FFA values when it is heated. Coconut milk is an emulsion of oil and water that is stabilized by protein (Srivastava & Semwal, 2015). The chemical composition of coconut milk was 2.6-4.4% protein, 50-54% water, 32-40% lipid and 1-1.5% ash (Seow & Gwee, 1997). The event of bad or 'off' flavor is caused by the advancement of free fatty acids resulting from the product of oil hydrolysis (Marina et al., 2009). Hydrolysis is quickened by lipase, which is normally found in tissue containing oils or lipids (Song et al., 2005.) Temperature is one of the components that affects lipase movement. The highest FFA and PV were found

at the accelerated storage condition (55°C) and the ideal temperature for lipase is in range of 30-40°C. (Sukasih et al., 2009).

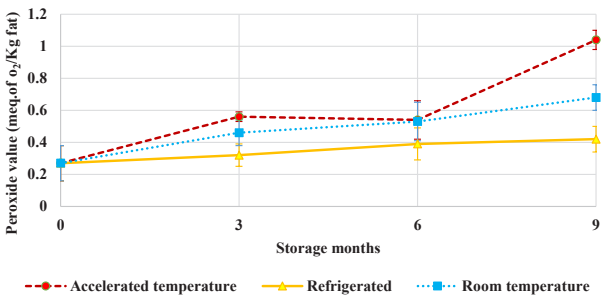

Fig. 5 Change in peroxide value (PV) content of RTE *Gulai-Besar* during different storage conditions up to 9 months.

3. Color

The color values of RTE *Gulai-Besar* under different storage period are presented in Table 4. The pigments, responsible for the color of the product, are affected by temperature conditions. A significant change is observed at the accelerate condition. The product had an initial L value of 44.58±0.10 which reduced dramatically to 32.21±0.20 on 9th month of storage time. Redness (a*) and yellowness (b*) were found to increase slightly with increasing storage time of all conditions. The red color is derived from the mixture of curry dried chili which is responsible for high a* value and the b* value occurred when coconut milk is heated. It changes the color to brown and/or darker (Tayeh, 2018). For determining the colorimetric indexes, the variation of the color was subsequently calculated in form of delta E (ΔE). The accelerate treatment showed a wide variation of parameters (ΔE) when compared to the other treatments, whereas the room storage treatment were observed with little variation in color, these variations were due to degradative processes.

4. Microbial quality

The higher heat resistance of many bacteria causes economical spoilage and disease. Total bacteria counts including anaerobic count, *Staphylococcal, Clostridium* spp., *E. coli* and yeast-mold were analyzed throughout the storage period but no microbial growth was observed in any sample. All microbes were below the approved food standards limit. It indicated that thermal processing led to commercial sterilization of the thermal process applied in processing of *Gulai-Besar* in retort pouches. The result coincides with the study of Chandirasekaran & Rajan (2016) who reported that no microbial growth was detected in retort pouch processed chettinad chicken during 180 days of storage time. Thus, it could be implied that the product was safe to consume.

5. Sensory analysis

The sensory of the RTE *Gulai-Besar* is shown in table 5. There was a slight difference in the sensory scores between refrigerated and room temperature, while accelerating temperature was noticeably decreased ($p<0.05$). The sensory attributes had a significant declined trend with an increase in storage time for all samples. However, the scores were within acceptable criteria in every sample. The lowest score of flavors occurred at the accelerated temperature (55°C) on 9th month of storage time. The decrease in sensory attributes might be due to the degradation of protein and oxidative changes in the product (Rajan et al., 2014).

Table 3 Chemical composition of RTE *Gulai-Besar* during different storage conditions up to 9 months

Parameters	Initial analysis	Storage period (months)								
		3			6			9		
		Storage condition								
		5 °C	RT	55 °C	5 °C	RT	55 °C	5 °C	RT	55 °C
Protein (%)	15.87±0.12	15.27±0.04^{a}	14.86±0.14^{b}	13.72±0.15^{c}	14.68±0.06^{a}	14.53±0.06^{b}	13.28±0.06^{c}	14.52±0.20^{a}	14.26±0.15^{a}	13.04±0.10^{b}
Fat (%)	9.23±0.21	9.23±0.010^{c}	9.81±0.09^{b}	10.08±0.09^{a}	9.88±0.12^{c}	10.08±0.03^{b}	10.72±0.02^{a}	9.94±0.08^{b}	10.15±0.10^{a}	10.93±0.19^{a}
Moisture (%)	67.94±0.15	66.55±0.14^{b}	65.93±0.14^{c}	67.27±0.04^{a}	65.13±0.09^{c}	66.90±0.10^{b}	67.63±0.15^{a}	65.01±0.10^{b}	65.80±0.06^{a}	65.74±0.12^{a}
Total ash (%)	2.58±0.02	2.62±0.02^{ns}	2.61±0.03^{ns}	2.66±0.03^{ns}	2.64±0.00^{b}	2.64±0.02^{b}	2.68±0.03^{a}	2.68±0.01^{b}	2.66±0.00^{c}	2.81±0.02^{a}
Carbohydrate (%)	4.38	6.33±0.02^{b}	6.79±0.02^{a}	6.27±0.03^{c}	7.67±0.01^{a}	5.85±0.02^{b}	5.69±0.02^{c}	7.92±0.01^{a}	7.13±0.02^{c}	7.48±0.01^{b}

Remark: Each value is presented as mean ± standard deviation (n=3), Different superscripts in the same row (different condition) indicate significant differences ($p<0.05$), ns shows that there was no statistically significant difference at ($p\geq0.05$).

Table 4 L*, a* and b* values of *Gulai-Besar* during different storage conditions up to 9 months

Parameters	Initial analysis	Storage period (months)								
		3			6			9		
		Storage condition								
		5 °C	RT	55 °C	5 °C	RT	55 °C	5 °C	RT	55 °C
L*	44.58±0.10	43.62±0.06^{a}	44.03±0.27^{a}	41.22±0.40^{b}	39.73±0.53^{b}	44.98±0.11^{a}	37.04±0.36^{c}	35.73±0.56^{b}	43.26±0.20^{a}	32.21±0.20^{c}
a*	12.45±0.04	12.26±0.03^{c}	13.95±0.14^{b}	14.83±0.23^{a}	9.56±0.46^{c}	12.91±0.60^{b}	17.34±0.58^{a}	8.12±0.60^{c}	12.80±0.54^{b}	17.22±0.16^{a}
b*	29.80±0.05	29.16±0.02^{c}	30.68±0.12^{b}	32.44±0.70^{a}	27.70±0.35^{c}	30.87±1.56^{b}	40.48±0.69^{a}	27.20±0.50^{c}	29.87±0.85^{b}	38.91±0.70^{a}
ΔE	0.00	1.17	1.15	4.89	6.02	1.23	13.96	11.62	1.37	12.88

Remark: Each value is presented as mean ± standard deviation (n=3), Different superscripts in the same row (different condition) indicate significant differences ($p<0.05$).

Table 5 Microbial changes of RTE *Gulai-Besar* during different storage conditions up to 9 months

Parameters	Initial analysis	Storage period (months)								
		3			6			9		
		Storage condition								
		5 °C	RT	55 °C	5 °C	RT	55 °C	5 °C	RT	55 °C
Total viable count (CFU/g)	<10^6	<10^6	<10^6	<10^6	<10^6	<10^6	<10^6	<10^6	<10^6	<10^6
Anaerobic count (CFU/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Staphylococcal (CFU/g)	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3
Coliform (MPN/100 g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Clostridium spp. (CFU/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
E.coli (MPN/g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yeast-mold (CFU/g)	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100

Remark: ND = not detect, each value is presented as mean ± standard deviation (n=3).

Table 6 Sensory evaluation results for RTE *Gulai-Besar* during different storage conditions up to 9 months

Parameters	Initial analysis	Storage period (months)								
		3			6			9		
		Storage condition								
		5 °C	RT	55 °C	5 °C	RT	55 °C	5 °C	RT	55 °C
Appearance	8.20±1.02	7.40±1.12^{ns}	7.45±0.08 ^{ns}	7.00±1.25^{ns}	7.46±0.10^{a}	7.22±0.14^{a}	6.27±0.89^{b}	7.20±0.13^{a}	7.00±0.21^{a}	6.00±0.72^{b}
Color	8.68±1.52	7.60±1.06^{a}	7.00±0.93^{ab}	6.60±1.24^{b}	8.27±1.10^{a}	7.53±0.83 ^{b}	6.20±0.68^{c}	8.00±1.24^{a}	6.95±0.08^{ab}	6.11±0.80^{b}
Flavor	8.67±1.40	7.67±1.45^{a}	7.27±1.10^{a}	6.87±1.85^{b}	7.07±1.22^{a}	7.12±1.20^{a}	5.80±1.32^{b}	7.02±1.50^{a}	6.98±1.12^{a}	4.52±0.68^{b}
Taste	8.94±1.15	7.94±1.44^{a}	7.60±1.06^{a}	6.80±1.66^{b}	7.47±1.46^{a}	7.54±1.52^{a}	5.73±1.03^{b}	7.31±1.02 ^{a}	7.20±1.00^{a}	5.65±1.08^{b}
Texture	8.78±1.08	7.80±1.42^{a}	7.80±1.18^{a}	6.80±1.47^{b}	7.73±1.71^{a}	7.40±0.94^{a}	5.70±1.10^{b}	7.56±1.48^{a}	7.34±0.45^{a}	4.89±1.90^{b}
Overall acceptability	8.80±1.00	8.27±1.10^{a}	7.67±0.83^{b}	6.67±1.68^{c}	7.67±1.45^{a}	7.53±1.40^{a}	5.60±1.12 ^{b}	7.63±1.42^{a}	7.50±1.40^{a}	4.90±1.16^{b}

Remark: Each value is presented as mean±standard deviation (n=3), Different superscripts in the same row (different condition) indicate significant differences ($p<0.05$), ns shows that there was no statistically significant difference at ($p\geq0.05$).

Conclusion

Gulai-Besar, a traditional meat product, was developed, packed and processed in retort pouch, and finally stored for 9 months in different conditions (refrigerated; 4±2°C, ambient temperature; 30±2°C accelerated; 55±2°C). The product was good sensory acceptability in all storage conditions with F_0 of 5.77.

All samples were microbial safe for 9 months. The present study suggested that retort pouch processing was suitable for 9 months storage of *Gulai-Besar* at ambient temperature with acceptable quality and safety. Thus, it would help to increase the market demand for traditional product due to convenience and ready to eat features.

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Expectancy on Being Older Persons and Self-Preparation for Old Age in Middle-Adults: Bang Phlad, Bangkok Metropolis

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Abstract

The purposes of this study were to assess the level of expectancy on being older persons and of the self-preparation for old age in middle-aged adults (ages between 35-60 years); and to estimate an equation to predict the self-preparation of middle-aged adults from the demographic factors and the expectancy on being older persons. The sample of 400 persons were recruited from middle-aged adults who were qualified with the predetermined criteria, using multi-stage random sampling technique. Data was collected using the questionnaire, developed by the researchers, consisted of 3 sections: the personal data, the expectancy on being older persons and the self-preparation for old age. The CVIs of sections 2 and 3 of the questionnaire were 0.97 and 1.0 with reliabilities of 0.92 and 0.93 respectively.

It was found that the expectancy of the sample on being older persons in general was at a high level (Mean 4.07, S.D. 0.66) and the self-preparation for old age in general was also at a high level (Mean 4.00, S.D. 0.55). The variables, which were analyzed using chi-square tests and Pearson's Product Moment Correlation test and found significantly associated with the self-preparation for old age, were later included to formulate a prediction equation of the dependent variable.

The Stepwise multiple regression analysis revealed the equation that best predicted the self-preparation for old age, with the Coefficient of determination (R^2) of 0.467 (R=.685). The equation included independent variables as follows: the expectancy on being older persons on spiritual and physical aspects, working for government/state enterprise/private company, practicing Buddhism, and having education at vocational certificate or higher levels. The implications of research findings and recommendations for future practice were discussed.

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Introduction

At present, world population aged 60 years and over has been increasing rapidly. Thailand is now an aging society, according to UN's definition, with an elderly population of about 11 million (16%) out of 63 million total population in 2018. Among all provinces in the country, Bangkok Metropolis has the highest number of elderly which accounts for 17.98 percent of total population (Department of Provincial Administration, Ministry of Interior, 2019). While the growth rate of country population is slowly increasing about 0.4 percent /year, but the population aged 60 and above has been increasing with the rate of 5 %/year, and the population aged 65 and above has been increasing even faster with the rate of 6 %/year (Foundation of Thai Gerontology Research and Development Institute, 2016). It was estimated that by the year 2021, Thailand will become complete aged society with the proportion of elderly population greater than 20% (Foundation of Thai Gerontology Research and Development Institute, 2017).

As a person becoming older, changes in various areas namely physical, psychological, emotional, social and economic aspects causing several problems. According to report on the Situation of the Thai Elderly 2017 (Foundation of Thai Gerontology Research and Development Institute, 2017), over 15% of the elderly reported that they had a poor or very poor health status. The most common health problems affecting older persons due to organ degenerations including cataracts, impaired hearing loss, impaired chewing ability due to tooth loss, osteoarthritis, dementia, and fall. According to the 5th national health survey by examination 2014 (Ekpalakorn, 2014), it was found that 22.3% of older persons had cataracts, 24.5% lived with impaired hearing loss, 52% had less than 20 teeth (permanent teeth and denture) causing impaired chewing ability, 22.5% with osteoarthritis, 8.1% with dementia, and 16.9 had a fall in the last six months. It was also found that some common chronic conditions frequently found among older persons were hypertension (53.2%), diabetes mellitus (18.1%), obesity (35.4%), abdominal obesity (49.4%) and metabolic syndrome (46.8%) (Ekpalakorn, 2014). These health problems are involved with one's behaviors since younger ages e.g. food consumption, exercise, smoking, alcohol drinking.

Such changes impair elderly's self-care abilities and become more dependent on family supports. Regarding to report on the Situation of the Thai Elderly 2017 (Foundation of Thai Gerontology Research and Development Institute, 2017), five percent of older persons cannot help themselves with daily life activities and the number has increased to 19% among late elderly (80 years old and over). Nowadays, family's potentials in providing care for older persons have been greatly compromised due to several factors. It was shown that extended families where older persons stayed with their families tend to decrease from 61.4% in 2003 to 58.3% in 2007. It was also found that the ratio of older persons living alone tend to be on the rise from 3.6% in 1994 to 6.3% in 2002, 7.7% in 2007 (National Statistical Office, 2013) and 11% in 2017 (Ekpalakorn, 2014). Similarly, the Potential Support Ratios which is defined as the number of people aged 15-64 per one older person aged 65 years and above are in the downward trend, having the ratio of 9.3 in 1994 to 7.0 in 2002 and 6.3 in 2007 (National Statistical Office, 2013). It was estimated that the ratio will be decreased to 3.8 in 2020 (Ekpalakorn, 2014). The current ratio can be interpreted that there will be less caregivers for each elderly since only four working-age persons will take care of each older person. The decline of the Potential Support Ratio would greatly impact the society in general since people in the working age had to share more burdens in caring for older persons. If this trend continues, both caregivers and older persons would be evenly weakened.

Problems concerning elderly have always been managed through reactive instead of proactive approaches with services provided to the elderly to address health problems and in response to elderly caring needs. Health promotion and disease prevention have already been realized as more effective approaches than curation and rehabilitation. Additionally, early investments by elderly themselves and the government have already been proved to be more cost effective. In practice, the preparation for ageing society should be started as soon as possible. Middle adulthood (ages 35-60 years) is considered a longer period when comparing to other age groups. This age group has the highest potential and productivity. People in this age group are adaptive and have ample life experience. If they realize the importance of preparation towards old age, it is most likely they will be able to adjust to the elderly well in both physical and other aspects. They will become independent and have good quality of life, matched with the concepts of "Active Aging" where persons and families realize and continuously gain responsibility in self-care (Nantsupawat, 2009). But the statistics showed that more

than 30% of Thai younger ages had not prepared themselves for good quality of old age in terms of health and financial security (Pooprasert, 2018).

Based upon the literature review, it was found that almost all studies on the adulthood's preparation to elderly were descriptive studies focusing on how persons prepare themselves for elderly as well as factors that played the role in the preparation process. For example Katisrivorapan (2008) explored psychosocial factors that related to retirement preparation behaviors among teachers ages between 45-59, while Pattrapakdikul, et al. (2011) examined factors associated with the preparations towards old age in 263 Faculty of Medicine of Prince of Songkla University's personnel aged 40 years and older. It was found from the latter study that age, marital status and salary were associated with preparation towards old age. In addition, other studies looked at elderly's and elderly-to-be's expectations. For instance, Meankerd examined value, elderly's expectations and age pluralism in a mixed-method of qualitative and quantitative study in both elderly and adult ages 15-59 years (all are called "plural age") in Bangkok and 16 other provinces (Meankerd, 2006). However, there is neither study that focused on expectations on being elderly nor which other factors would play roles in the self-preparation for old age in middle-adults. This reflects the importance of conducting a study on expectancy and self-preparation for old age in middle-adults in comprehensive aspects.

Bang Phlad district was selected as the study site because of the location of the Faculty of Nursing. The Faculty has been working closely with the community through the students' practice in Community Health Practicum courses. This area is determined by the Faculty to be learning resources for faculties and students as well as a location where the faculty can provide academic services. Furthermore, understandings from this study would provide as an input for the Bangkok Municipality or other related agencies to use as an input for policy setting and strategic planning for aging population.

The objectives of this study were to explore the levels of expectancy on being older persons and self-preparation for old age in middle-adults. Its objective was also to test the relationships between demographic factors, expectancy on being older persons and self-preparation for old age. Vroom's Expectancy Theory (Management Study Guide Website, 2016) which states that persons would gain motivation toward certain behavior when they expect the results or rewards that would follow such behavior and whether the results or rewards had high impact to them, was used as the framework of this study. The hypothesis of this study was that expectancy on being older persons and that demographic factors were associated and able to predict self-preparation for old age in middle-aged adults.

Methods

The population in this study were middle-aged adult ages between 35-60 years residing in Bang Phlad district, Bangkok. According to statistics on population and household by age groups in Bang Phlad district, Bangkok in December 2013, there were the total population of 39,200 persons middle-aged adults (Department of Provincial Administration, Ministry of Internal Affairs, 2016). The whole population could be divided to 10,016, 10,364, 8,111 and 10,709 persons from Bang Phlad, Bang or, Bang Bamru and Bang Yi Khan sub-districts, respectively. The sample determination was done using multi-stage sampling through the use of Glenn D. Israel's table (Israel, 2009) where the population was between 25,001-50,000 with 5% errors (95% confidence level) yielding the sample size of 397. The sample within sub-districts were calculated using the population proportion yielding the sample size of each sub-district as follows: Bang Phlad = 101, Bang or = 105, Bang Bamru = 82, and Bang Yi Khan = 109 persons.

1. Scope of the study

This study aims at exploring middle-aged adults' expectations and self-preparation towards elderly. The inclusion criteria of the qualified samples included being Thai citizen, ages between 35-60 years old, able to read and write Thai language, fully orientation, residing in Bangkok for five years or more, and are in good health. The study exempted persons with disabilities that require special assistances from other people to be included in the samples. The study was conducted during October, 2015 and October, 2016. It was approved by the Institutional Review Board (IRB) under Suan Dusit University Institute of Research and Development (Approval number SDU-RDI 2015-006).

2. Definitions

In addition to the aforementioned Vroom's Expectancy Theory, the conceptual framework of this study was formulated by looking how people would expect the wellness of their life should be when they become old age. According to the National Wellness Institute, wellness is defined, "an active process through which

people become aware of, and make choices toward, a more successful existence" (National Wellness Institute, 2019). Dr. Bill Hettler, co-founder of the National Wellness Institute encompassed six dimensions of wellness namely:-emotional, occupational, physical, social, intellectual and spiritual; while some others comprise wellness as many as eight dimensions i.e. occupational, emotional, spiritual, environmental, financial, physical, social, and intellectual (Stoewen, 2015). Wellness and health are terms that are often interchanged but they are not the same. Health is a state of being, whereas wellness is the state of living a healthy lifestyle. Health refers to physical, mental, and social well-being; while wellness aims to enhance well-being (University of California, Davis Campus, 2019). In this study, we considered that when people expected how their life in old ages should be, they would have a motivation to take action to prepare themselves. To make it simple for ordinary people, we grouped the self-preparation process for old age into four dimensions i.e. physical, psychological, spiritual and socio-economic aspects. Extensive search was done to get the information on how middle-aged adults should prepare themselves on each aspect to achieve their wellness. Research instruments were then developed by researchers according to the concepts and definition stated below.

2.1 Self-preparation for old age is defined as issues that persons in middle adulthood (ages 35-60 years) have realized, prioritized or attempted to seek for information, measures and put into actions, training, or practices in physical, psychological, spiritual, economic and social dimensions in order to help them make a smooth transition to be older persons with happiness and quality of life according to the persons' potentials and life contexts.

2.2 Physical preparation includes person's activities involved with nutrition, exercise, resting and sleeping, personal hygiene, health information seeking, regular check-up, self-care while being sick and accident prevention.

2.3 Psychological preparation includes person's activities involved with having good mental health, positive attitudes, good relationships with people, effective stress management and emotional control and conflict resolution, love and respect in oneself and others, self-confidence, self-responsibility, flexible personality and easily approachable, open-minded and ready to listen to other's opinions and understanding other's feelings.

2.4 Spiritual preparation includes person's activities involved with seeking for the meaning of life, going to Buddhist temples, churches, mosques or other places to practice religious or faith activities, reducing selfishness through giving and providing community services, forgiving and being ready to apologize, practicing and developing oneself through learning and contemplating on events that occur to oneself or observe from others' experiences, facing life's problems and difficulties without being despair while maintaining hope, being able to detach to problems or troubles that are greater than one's capabilities.

2.5 Socio-economic preparation includes person's activities involved with having jobs or professions that yield income and life security, saving for future, maintaining good relationships with one's family, relatives and neighbors, participates in community and social networks, learning technologies for communications; and being ready for the changes of the society.

2.6 Expectancy on being older persons in this study includes what persons in middle-age expect when they become elderly such as health conditions, the care they may need from families, relatives, community or social groups they belong to, as well as government and related agencies regarding to physical, psychological, spiritual and socio-economic aspects.

3. Research conceptual framework

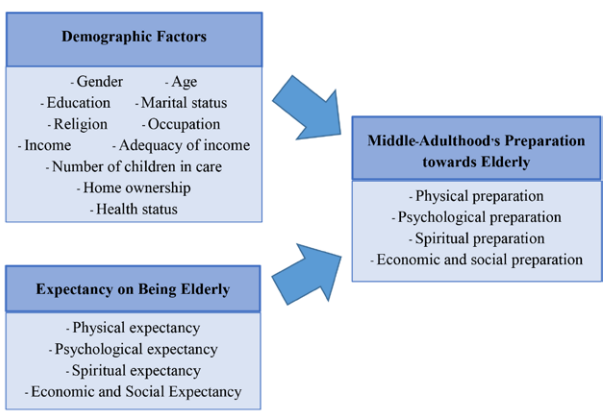

Fig. 1 Conceptual framework of this study.

Based on the literature reviews, the conceptual framework of this study included independent variables as follows: 1) Demographic factors: gender, age, education, marital status, religion, occupation, income, adequacy of income, number of children in care, home

ownership and health status; 2) Expectancy on being older persons on physical, psychological, spiritual and socio-economic aspects. The independent variables were examined whether they are associated with the dependent variable of the study: Self-preparation for old age which also included physical, psychological, spiritual and socio-economic aspects.

4. Research instrument development and validation

The instrument used in this study was a questionnaire developed by the researchers based on theoretical concepts from the literature review. The instruments were consisted of 3 parts: 1) general information of the respondents in which consisted of 16 multiple-choice items; 2) expectancy on being older persons in physical (8 items), psychological (5 items), spiritual (6 items) and socio-economic (5 items) aspects totaling 24 five-level rating scale questions; and 3) self-preparation for old age covering physical (16 items), psychological (16 items), spiritual (10 items) and socio-economic (12 items) aspects for the total of 54 five-level rating scale questions. The instrument was tested for face and content validity through the revisions of three experts in adult and elderly care and measurement and evaluation, yielding the content validity index (CVI) from expectancy on being older persons and self-preparation for old age parts of 0.97 and 1.0 respectively. The tests for reliability with 30 respondents who possessed equal characteristics as the samples in this study showed high reliabilities with Cronbach's alpha coefficients from those two parts of 0.92 and 0.93 respectively.

The sampling process in this study was done using multi-stage sampling techniques by randomly selecting 3 communities from each of four sub-districts. Families in each selected community were then systematically identified through consultations with community leaders or Bangkok metropolitan volunteers, after asking permission to collect the data from adult family members that met the selection criteria. The researcher drew a name in-case there were more than 1 adult representing each family, so that only one respondent would represent each family. Data was then collected from each respondent who agreed to participate using the questionnaire according to the protocol on human subject protection approved by the Institutional Review Board (IRB). There was a total of 400 respondents in this study with 101 persons from Bang Phlad, 108 persons from Bang or, 82 persons Bang Bamru and 109 persons from Bang Yi Khan sub-districts.

5. Data analysis

5.1 The descriptive information from the study including demographic data, expectancy on being older persons and self-preparation for old age were analyzed both itemized and as a whole using percentages, means and standard deviations. The interpretation criteria in self-preparation for older age are as follows:

- Average scores between 4.51 and 5.00 were interpreted as the highest level of self-preparation with activities being practiced regularly.
- Average scores between 3.51 and 4.50 were interpreted as a high level of self-preparation with activities being practiced quite regularly.
- Average scores between 2.51 and 3.50 were interpreted as moderate level of self-preparation with activities being practiced occasionally.
- Average scores lower than 2.50 were interpreted as low level of self-preparation with activities being practiced rarely or no activity at all.

For the expectancy on being older adults score, shall be interpreted in the same way that: the average scores between 4.51 and 5.00, 3.51 and 4.50, 2.51 and 3.50, and lower than 2.50 were interpreted as the highest, high, moderate, and low levels of expectation or care needed respectively.

5.2 The associations between personal information and expectancy on being older persons and self-preparation for old age were done using chi-square and Pearson's Product Moment Correlation Coefficients.

5.3 Predictive equations were formulated from the set of independent variables that are significantly associated with the dependent variable using multiple regression analysis.

Results and discussion

1. Demographic information

The majority of the samples were female (63.7%) with ages ranged from 35-60 years old (Mean = 47.9 years, SD = 7.55) with reported marital status as married (62.3%), practiced Buddhism (85.0%), graduated from elementary schools (26.8%), and had no income or income lower than 10,000 Baht per month (32.6%). When comparing incomes and expenses, the majority of the samples had adequate incomes but had no saving (42.8%) followed by those who had inadequate income with debts (18.8%). Most of them had children (71.4%) while the majority reported having only one child (32.3%). The samples lived in the same households with their parents

(39.8%) and owned their own home (31.8%). Almost half of the samples were in overweight status (BMI between 25.0-29.9 kg/m^2) (31.1%) and obesity status with BMI exceeding 30.0 kg/m^2 (14.6%). More than half (61.4%) did not have chronic illness. Hypertension (11.0%) and diabetes mellitus (6.0%) were more prevalent among those with chronic illness. Persons with more than one illness accounted for 6.8% of all samples. The majority of samples with chronic illnesses received treatments through contemporary medicine (89.7%). Regarding the Health Insurance Programs for which the samples eligible, it was found that 43.7% were covered by the National Health Security Program (NHSP), while 29.1% were eligible for the Social Security System (SSS).

2. Expectancy on being older persons

According to table 1, the samples' overall expectancy on being older persons was at a high level with the highest on physical and psychological aspects while socio-economic aspect being the lowest among all. Item with highest average score in physical expectancy aspect and appeared to be the item with the highest average score overall was "the ability to help oneself with personal daily activities (Mean = 4.54, SD = 0.78). Item with the lowest average score in this aspect was "receiving government/non-government funded housing for elderly. This showed the samples' expectation to being able to help oneself with their daily activities and become less or no burden to their children. Their responded of having their own children taking care of them at home (Mean = 3.82) was higher than the item of staying in special housing for elderly (Mean = 3.02). Teewunda & Sanjai (2010) and Rittirong et al. (2014) also found that elderly who needs assistances would prefer receiving assistances from their children followed by their grandchildren, before receiving such assistances from other people or entities. According to the psychological expectancy aspect, it was found that the item with highest average score were "having good mental health" (Mean = 4.37), followed by "being able to help others and were viewed as being helpful for their children" (Mean = 4.30), and "receiving love and care as well as respect from their children" (Mean = 4.27). Item with lowest average score was "being carefree with no worry or concerns with any issues." This showed that the samples placed their priority on having good relationships with their children and realistically looked at their future that they might eventually have to confront situations that cause some worries and concerns. According to the spiritual expectancy aspect, items with highest average score was "having spiritual anchor or stronghold that enable oneself to withstand when confront with problems or life's difficulties (Mean = 4.25, SD = 0.84). Three items receiving lowest average scores were "being prepared to appropriately confront illness and the end of life" (Mean = 4.02), "having a peaceful mind" (Mean = 4.10), and "receiving care at the end of life with pride and dignity" (Mean = 4.10). Such findings could be explained that, since the samples were at the middle adulthood with good health and ability to provide for their families, it was possible the end-of-life preparation might not be as yet of their concerns. According to the socio-economic expectancy aspect, item with the highest average score was "receiving news about politics or being able to learn the issues subjected to their interests" (Mean = 4.08, SD = 0.94). Item with lowest average score was "having adequate income to maintain their lives after 60 years of age" (Mean = 3.49, SD = 1.33). This item appeared to be the lowest among all 24 items. It showed from the findings that the samples, while expected to be able to assist themselves, physically, expected to have adequate income after retirement at age 60 years at only an average level.

3. Self-preparation for old age

Based on table 2, the samples' preparations for old age both in each aspect and overall were at a high level, ranking from the highest to the lowest levels of preparations as follows: psychological, spiritual, socio-economic, and physical aspects with average scores of 4.30, 4.17, 3.95, and 3.68 respectively. Based on each item, two items with the highest preparation level were "trying to depend on oneself before others" (Mean = 4.56) and "knowing one's responsibility while not placing responsibility to others" (Mean = 4.52). Both items were in psychological aspect, showing the consistency in the findings of their expectancy on being older persons where the samples expected that they could do their personal daily activities by themselves without being burden to their children.

Table 1 Expectancy on being older persons

Aspects of Expectancy	N	Expectancy on Being older persons		
		Mean	S.D.	Level
Physical Expectancy	381	4.23	0.74	High
Psychological Expectancy	397	4.23	0.75	High
Spiritual Expectancy	393	4.15	0.72	High
Socio-Economic Expectancy	396	3.86	0.87	High
Overall Expectancy	**373**	**4.07**	**0.66**	**High**

Table 2 Self-preparation for old age

Aspects of Self-Preparation	N	Self-Preparation for old age		
		Mean	S.D.	Level
Physical Preparation	373	3.68	0.61	High
Psychological Preparation	386	4.30	0.61	High
Spiritual Preparation	385	4.17	0.67	High
Socio-economic Preparation	392	3.88	0.71	High
Overall Preparation	**344**	**4.00**	**0.55**	**High**

Upon analyzing each aspect, it was found that 3 items with the lowest average score in physical aspect included "exercising at least 3 days per week and at least 30 minutes each day" (Mean = 2.86), "avoiding high fat diets" (Mean = 3.15), and "controlling oneself from becoming abdominal obesity (Mean = 3.18). According to the psychological preparations, all items were at a high level except for 2 items regarding "self-reliance" and "understanding their own responsibilities" having a very high level. Regarding the spiritual preparation, all items were at a very high level. It was important to note that items dealing with preparations within oneself showing spiritual and wisdom development such as "living a meaningful life" and "confronting hardships without despair" had higher average scores than items asking about outward behaviors such as "going to Buddhist temples, churches, or Mosques" or "practicing activities based on beliefs and faith in their religions." Regarding to the socio-economic preparations, it was found that "having occupations that can generate incomes", "preparing housing," "managing appropriate time among works, family and community," and "being able to catch up with societal changes were items with high average scores." Items with lowest average score was "preparing money for the future" (Mean = 3.46, SD = 1.46).

Based on the findings, issues required to be promoted among middle-age adults, in order to make a smooth transition to their elderly, in physical aspect included exercises, avoiding high fat food, and controlling the abdominal obesity; in socio-economic aspect included preparing money for their retirement through saving, provident fund, National Saving Fund, Long Term Equity Fund (LTF), Retirement Mutual Fund (RMF), life insurance, etc. Aging is a long process starting even before a person was born and occurred throughout the life. Health status and functional abilities during aging were the accumulation of the person's lifestyles and activities that occurred previously (Rittirong et al., 2014). According to the Life Course Theory, (Marriage & Family Encyclopedia, 2016; Hutchison, 2001) it was the past that shapes the future and could be explained that what happened at the early age whether decision makings, opportunities, or life conditions would cumulatively affect the rest of one's life. Therefore, it is recommended to make adjustment on lifestyles that conducive to health at all ages. One of the myths about aging is that it is always too late making lifestyle change during old age. The fact is that lifestyle changes, even when they begin during old age elderly would help prevent diseases and degeneration of body functions. This will eventually help prolong life as well as increase quality of life (World Health Organization, 2002). Lifestyle change if started early as a person is in adulthood, would definitely yield benefits to the person. As for the item on "preparing money for the future" which got the lowest score was consistent with the national statistics mentioned before that more than 30% of Thai younger ages had not prepared themselves for quality old ages in terms of health and financial security (Pooprasert, 2018). This issue needs proper social interventions to raise awareness and provide knowledge or advice to middle-aged adults so that they can make saving plans to suite with their own life.

4. Association testing among variables

Based on table 3, independent variables that were found significantly associated with adults' preparations for old age at 0.05 significant level, according to Chi-square test, included gender, educational level, marital status, religion, occupation, salary, adequacy of income, and home ownership with p-value < 0.05. Based on the correlation tests using Pearson's Product Moment Correlation, variable that significantly associated with adult's preparations for old age was the adult expectancy on be older persons ($r = 0.648$, p-value < 0.01). Therefore, all independent variables were found to be significantly associated with the dependent variable except for health status, age, and number of children in care. Those would be later included in multiple regression analysis.

Findings from this study supported the findings from 2011 study conducted by Pattrapakdikul et al. (2011). Factors namely:- age, marital status and income were found associated with preparation toward elderly among 263 personnel aged 40 years and older from Faculty of Medicine at Prince of Songkla University. Another study by Ratchaneeladdajit (2012) examined food consumption patterns, nutritional status, health-related quality of life, and preparation towards elderly among 230 teachers ages 40-59 years old from Kanchanaburi educational service area 1 and found that gender and age group were significantly associated with person's preparation toward elderly at 0.05 significant level. It was also found that

Table 3 Test results of associations among variables

Variables	Preparation Levels						X^2	P-value
	Low/ Moderate		High		Very High			
	n	%	n	%	n	%		
Gender							6.599	0.037*
Female	37	16.7	133	60.2	51	23.1		
Male	17	13.8	90	73.2	16	13.0		
Education							28.126	0.000**
None (in school system) & Primary	27	27.0	62	62.0	11	11.0		
Secondary	15	17.0	62	70.5	11	12.5		
Diploma / Certificate and higher	12	7.7	99	63.5	45	28.8		
Marital status							12.599	0.013*
Single	14	19.2	44	60.3	15	20.5		
Married	24	11.2	151	70.2	40	18.6		
Widowed / Separated	16	29.1	28	50.9	11	20.0		
Religion							12.158	.002**
Buddhism	52	17.7	193	65.6	49	16.7		
Islam	2	4.1	30	61.2	17	34.7		
Occupation							20.750	0.000**
None / Daily Worker	28	24.1	74	63.8	14	12.1		
Government / State enterprise / Company employee	8	6.9	74	63.8	34	29.3		
Merchant / Business owner / Self-employed	18	16.4	74	67.3	18	16.4		
Salary (Baht/Month)							15.901	0.044**
0-10,000	28	24.1	70	60.3	18	15.5		
10,000-20,000	16	14.0	78	68.4	20	17.5		
20,001-30,000	6	12.8	33	70.2	8	17.0		
30,001-40,000	2	6.5	19	61.3	10	32.3		
>40,000	2	5.7	23	65.7	10	28.6		
Adequacy of Income							13.532	0.009**
Adequate with savings	17	12.8	77	57.9	39	29.3		
Adequate without savings	25	17.4	99	68.8	20	13.9		
Inadequate and have debts	12	18.2	46	69.7	8	12.1		
Home ownership							15.607	0.000**
Yes	30	11.7	169	65.8	58	22.6		
No	24	27.6	54	62.1	9	10.3		
Health status							0.158	0.924
No illness / Healthy	32	15.2	138	65.4	41	19.4		
With illness / Not healthy	22	16.7	84	63.6	26	19.7		

	N	R	P -value
Age	400	0.032	0.558
Number of children in care	396	.007	0.902
Expectancy on being elderly	328	0.648	0.000**

females were 2.6 time better preparing for old age than their male counterparts while older persons were 3 times better preparing for old age than their younger counterparts. However, health status, age and number of children in care were found not significantly related; possibly due to a multi-dimensional nature (included all physical, psychological, spiritual and socio-economic aspects) of the definition of self-preparation for old age. In order for the variables to be significantly associated in the study, all aspects must be associated simultaneously. Therefore, in the current study, the researchers further examined the associations between health status, age and number of children in care and self-preparation for old age in separate aspects. It was found that age was significantly associated with self-preparation for old age in physical aspect only (r = 0.122, p-value = 0.019). No significant association was found between health status and number of children in care with self-preparation for old age in any of four aspects.

5. Equations predicting "Self-preparation for old age"

In creating predictive equation from the set of independent variables that associated with the dependent variables (Table 3) using multiple regression analysis, the following steps were followed:

5.1 Preliminary tests including Normality test, Linearity and Homoscedasticity of Homogeneity of variance were conducted. Results from Normality test using graph plotting and Kolmogorov-Smirnov test showed that one independent variable (Expectancy on being older persons-SExp) and the dependent variable (Self-preparation for old age-SP) were not normally distributed. Both variables were then transformed using logarithms, square roots and inverse formations to correct such non-normal distributions (Hair et al., 2010). Square root method was selected for it best provided normal distribution of both variables and met assumptions for multiple regression including normality, linearity, and homoscedasticity

5.2 Variables with nominal scale were transformed to dummy variables (Tirakanant, 2012). There were the total of 12 variables to be tested in the multiple regression while there were 325-364 valid cases yielding the proportion of valid samples: number of independent variables = 27:1 to 30:1. According to Hair et al., the lowest acceptable proportion was 5:1. It was also recommended that, in order to make a confidence reference to the whole population, proportion of sample size and independent variable should be 15-20 : one independent variable (Hair et al., 2010).

5.3 The equation that best predict dependent variable was selected using stepwise regression with Multicollinearity, Independence of Errors and Outliers. Table 4 showed the results from multiple regression in creating an equation that best predicted Self-Preparation for old age (sqr SP).

Table 4 showed the best predictive equation when using the expectancy on being older persons in each aspect namely; physical (sqrSPE), psychological (sqrSPsE), spiritual (sqrSSE), and socio-economic (sqrSEE) expectancies as the independent variables instead of the overall expectancy. It was found that all variables in the equation were able to predict about 46.90 percent of the self-preparation for old age ($R = 0.685$, $R^2 = 0.469$). Independent variables included in the equation were Spiritual Expectancy (sqrSSE), Physical Expectancy (sqrSPE), being government officers or business employees (OCC2), practicing Buddhism (RELIGION1), and having education at diploma/certificate levels or higher (EDU3).

Table 4 Self-preparation for old age: Multiple regression analysis results

	Unstandardized Coef.				
Variable	B	SE	Beta	T	Sig.
Constant	.762	.049		14.893	.000**
sqrSSE	.339	.036	.470	9.346	.000**
sqrSPE	.154	.037	.210	4.133	.000**
OCC2	-.051	.019	-.128	-2.745	.006**
RELIGION1	.055	.022	.104	2.521	.012*
EDU3	-.036	.018	-.095	-2.031	.043*
R = .685	Adjusted R^2 = .461		Durbin-Watson = 1.811		
R^2 = .469	SE = .138		Std. Residual Min= -3.014, Max =2.602		
Source of variance	SS	Df	MS	F	Sig.
Regression	5.410	5	1.082	56.639	0.000**
Residual	6.113	320	.019		
Total	11.523	325			

Remark: * Statistical significant at .05 level
** Statistical significant at .01 level

The multiple regression analysis in this study met all criteria including no Multicollinearity among variables (with Tolerance values of all variables > 0.10 and VIF < 10) (Tirakanant, 2012). Durbin-Watson Test was 1.811 showing no auto-correlation among variables. Scatter plot of standardized residual were Null plot showing that the error values were random or were independent to each other. The Normal probability plot was in diagonal line showing the normal distribution of the error values (Hair et al., 2010). Additionally, standard residual of the self-preparation for old age (sqrSP) were between -3.014 and 2.602, showing no outliers (Tirakanant, 2012).

The predictive equation from the standardized scores was as follows:

$$ZsqrSP = 0.470\ sqrSSE + 210\ SPE - 0.128\ OCC2 + 0.104\ RELIGION1 - 0.095\ EDU3$$

The equation showed that both Spiritual Expectancy on being older persons (sqrSSE) and Physical Expectancy on being older persons (sqrSPE) influenced the self-preparation for old age of middle-age adults. Given the positive standardized coefficients (Beta), it can be further explained that persons with higher spiritual and physical expectancies will have greater preparations toward elderly. Given the negative Betas, persons working for the government, state enterprise, or private companies will have fewer preparations toward elderly, comparing to other professions. Persons who practice Buddhism will have greater preparations for old age than persons in other religions/faiths. Persons graduated from diploma/certificate levels will have fewer preparation toward elderly when comparing to those graduated from primary or secondary levels.

Results from these analyses explained that the expectancy on being older persons, especially spiritual and physical aspects had greatest influences on middle-age adults' preparation for older persons, with the adjusted R^2 = 0.417 meaning both variables could predict the variation of the self-preparation for old age up to 41.7 percent. When dummy variables (occupation, religion and education) were included into the equation, the adjusted R^2 only increased by only 5%, yielding the final Adjusted R^2 of 0.461. The findings confirmed Vroom's Expectancy Theory where persons would gain motivation toward certain behavior when they expect the results or rewards that would follow such behavior and whether the results or rewards had high impact to them. Therefore, it can be concluded that middle-age adults' expectancy on spiritual and physical aspects influenced their preparation toward elderly.

In order to gain more understanding on how those demographic variables of middle-age adults influenced their self-preparations, the researchers created predictive equations on self-preparation for old age in each aspect, namely physical, psychological, spiritual and socio-economic aspects. The findings reveal that dummy variables on having no or primary educational level, and practicing Buddhism had positive Betas, while variables on having at least diploma/certificate educational level, working in civil services/state enterprise/private company, owning a house, and being married had negative Betas in predictive equations. These results could be explained using Maslow's Hierarchy of Needs Theory, where human beings' needs starting from basic and increasing once their needs at the lower levels have been fulfilled (Maslow, 1954). Human needs start from physiological needs and advance to safety needs, belonging and love needs, esteem needs, and self-actualization being the highest level. It can be explained that people with limited education as well as income may not meet their basic needs, therefore would put more efforts in preparing themselves for changes such as becoming older persons. Persons with higher educational levels, as well as persons with more secure jobs (working for civil services/state enterprise/private company), owning the house, and being married were the group whose basic needs have been met, requiring fewer preparation toward elderly when comparing to the former group.

The findings also showed that persons practicing Buddhism better prepared themselves toward elderly

than those being Muslim (There were only 2 dummy variables: Buddhism and Muslim). It could be explained from the principles of Buddhism to always "being independent and try to help oneself as much as possible." This also confirmed with their response where their expectations in helping themselves in personal daily activities was the item with the highest score.

6. Implications and Recommendations for Future Practice

6.1 Based on the findings from the study on self-preparation for old age, items with lowest average score reflected the needs to promote their preparation to become quality elderly or active ageing. On physical aspect included exercising, avoiding high fat food, controlling their weight and preventing abdominal obesity and on socio-economic aspect included making financial plans for the future. When realizing that middle-age adults wanted to become self-reliance especially being to do their own personal activities when reaching elderly, it is very important for persons to start paying more attention to their health as early as possible.

6.2 Expectancy on being older persons, especially on spiritual and physical aspects had highest influences on their preparations; it can be used as a guideline in building concerns among adults in order to make a smooth transition toward elderly.

6.3 The predictive equation showed that persons with limited education had better preparation toward elderly than persons with higher educational levels while persons working for civil services/state enterprise/private company had lower level of preparation toward elderly. This finding can be useful to choose target groups in building awareness in preparation toward elderly.

6.4 The results of this study were based on one site study, it needs a careful consideration to apply to other constituencies. Therefore, further investigation is recommended.

Conclusion

This study represents an attempt to explore middle-aged adults' expectations and self-preparation towards elderly in comprehensive aspects. The study showed that although the expectations and self-preparation of the sample were both in high level in general, some issues still required to be promoted among middle-age adults in order to make a smooth transition to their elderly. It was also found that middle-age adults' expectancy especially on spiritual and physical aspects influenced their preparation toward elderly. Therefore, we concluded that middle-age adults' expectancy influenced their preparation toward elderly and that the findings supported Vroom's Expectancy Theory.

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Mentum Deformities of Chironomid Larvae in Huai Kakang Stream (Northeastern Thailand) and Lead Exposure Effect

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Abstract

Deformities in the mouthparts of Chironomidae larvae, particularly of the teeth on the mentum, have been proposed as a bioindicator of anthropogenic impacts on water quality. Therefore, this study was used the deformities of Chironomid larvae for assessing water quality of Huai Kakang stream (Maha Sarakham Province, Thailand). The effect of lead exposure in the mentum deformities of *Chironomus* spp. was also studied to confirm the relationship between the toxicant and the deformities. Water, sediment and chironomids larvae, were collected from Huai Kakang stream. Water and sediment were analyzed for nitrate (NO_3^-), total phosphate (TP), chemical oxygen demand (COD) and heavy metals (i.e. Cd, Cu, Ni, Pb and Zn). Chironomids larvae were examined for the mentum deformity. For the bioassay test, the larvae were raised in 4 different concentrations of lead, including 0, 10, 20 and 30 mg_{Pb}/L. The results indicated that the lower stream site of Huai Kakang was polluted with the exceeding limits of Lead and cadmium. The 53.33% of chironomid larvae exhibited mentum abnormality. The bioassay test showed the high concentration of lead caused the larvae to reduce in number. The 50% lethal concentration (LC_{50}) was 34.74 mg_{Pb}/L. The experiment of 20 mg_{Pb}/L showed the highest percentage of deformity and bioaccumulation of chironomids.

Introduction

Most water quality monitoring tools employed in Thai legislation are mainly along Physicochemical parameters, with the exception of *Escherichia coli*. However, environmental protection agency and academic intuitions are working on using biological indicators in biomonitoring programs in the country. Most biological water quality monitoring tools employed in Thailand are based on community response to water quality impairments. However, community structure assessments are essentially measures of lethality and concentrations of contaminants must be high enough to result in the disappearance, or reduced abundance and diversity of sensitive taxa before the community response approach would detect water quality stressors (Odume et al., 2012). The morphological deformities in aquatic animals such as the gill abnormality of hydropsychids

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larvae (Vouri & Kokkonnen, 2002; Prommi & Thamsenanupap, 2013), or mouthpart deformity of chironomids larvae (Al-Shami et al., 2011; Odume et al., 2012; Thani & Prommi, 2017) were used and represent sub-lethal response to in-term pollutants and are considered early warning indicators of water quality deterioration.

The Chironomidae are considered to be the ideal bioassay organisms since they spend most of their life stage in water where they are exposed to toxicants in both water and sediments. When continuously exposed to the stress of pollution, late instar of chironomid larvae frequently develop deformities in their mouthparts, especially mentum (Al-Shami et al., 2011). Therefore, the application of chironomids' mentum deformity as bioindicators of pollution stress have been published and illustrated primarily for bioassessment purposes.

In Thailand, a few papers on mentum abnormality of chironomids were published, such as Thani & Prommi (2017) which studied the use of mentum deformity of Chironomidae to indicate environmental perturbation in freshwater habitats. No published paper was studied on the toxicity test. Therefore, this paper aims to 1) study the effect of anthropogenic impacts on the Huai Kakang stream based on the mentum deformity in Chironomidae and 2) conduct an experiment on lead exposure in *Chironomus* spp. and their effects on the mentum deformities. The experiment was conducted to clarify that the deformity caused by the pollutants was found in the site's survey.

Materials and methods

The research was conducted for 2 studies. The first study was conducted to survey the deformity of chironomids' mentum and together with water quality parameters to assess Huai Kakang stream quality. The second study was an experiment on lead exposure of *Chironomus* spp. The latter study was conducted in a purpose of explanation of mentum deformity and metals concentration relations. Details of methodology were as follows.

1. A survey on mentum deformities of *Chiromomus* spp. in Huai Kakang stream

1.1 Site descriptions

Huai Kakang stream has an estimated 43 km length, and originates from Bann Kok Koh, Maha Sarakham Province and flows to Chi river at Roi-et Province, Thailand. This stream is the main water resource for local people for many activities, including agriculture, aquaculture, recreation and for receiving wastewater. In this study, the sampling sites were two sites at Haui Kakang stream, Maha Sarakham Province (Fig. 1). The first site represented the upper stream site, where was chosen for a reference site. This site is located at the coordination of 16°04'26.4"N 103°15'39.7"E, where is surrounded by agricultural area and small villages. The second site is the lower stream site, where is located at the coordination of 16°11'26.4"N 103°18'27.1"E. This site has directly received untreated community wastewater from Maha Sarakham municipality. Therefore, this site was chosen for an impacted site.

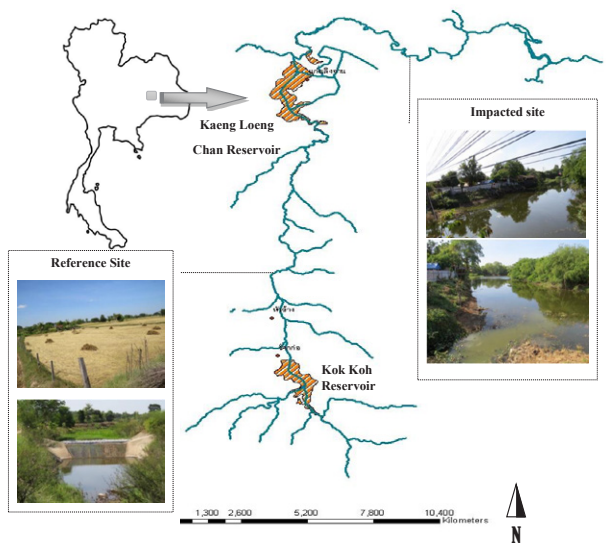

Fig. 1 Map of sampling site located at Huai Kakang stream in Maha Sarakham Province, Thailand.

1.2 Field sampling and investigate

At each site, water was sampled using water sampler and placed in polyethylene bottle and refrigerated at 4°C. Physicochemical parameters were analyzed in laboratory at Faculty of Environment and Resource Studies, Mahasarakham University, comprised with nitrate (NO_3^-), total phosphate (TP), chemical oxygen demand (COD), and heavy metals (i.e. Cd, Cu, Ni, Pb and Zn) (APHA, 1992).

Sediment sample was randomly collected for 1 kg. at each site. The samples were air dried, ground and sifted using 0.2 mm. sieve in the laboratory and prepared for analyzing heavy metals (i.e. Cd, Cu, Ni, Pb and Zn) using Flame Atomic Absorption Spectrophotometer (AAS).

After finished water and sediment sampling, the chironomid larvae were also randomly sampled by hand picking technique in distance of 10-20 meters'of the stream's reach. A D-frame aquatic dip net was used for collecting the larval samples in a predominant find sand and silt habitats. The sampling was conducted to find as much as targeted chironomids in at least 30 minutes of investigate. The chironomid larvae were kept in 70% EtOH in glass vessel.

Each chironomids was transferred to a standard glass microscope slide. A head capsule was cut under the microscope and keeping the head capsule wet at all times by adding a drop or two drop of ethanol. The head capsule was transferred to 10%(w/v) NaOH in a beaker and soaked for 10-30 mins depending on size or recognized clear sample. The sample was move to slide with a drop of mounting media (i.e. resin) and then covered by a slip. Chironomid specimens were identified according to the number of ventral and/or anal tublues, setae, parapodia, paraligula, ligula and metum follow Simwiset et al. (2015). All menta were examined and evaluated for the presence of deformities and labeled. The type of deformity established according to the classification by Lenet (1993) and Reynolds & Ferrington (2001). Water, sediment and chironomid larvae were sampled 2 times during January and February 2016.

The incident of physicochemical parameters and mouthpart deformity of Chironomids was expected. The confirmation of this relation, laboratory bioassays was needed. Lead was the highest amount toxicant found exceeding the standard limits. Consequently, the experiment was on the exposure of *Chironomus* spp. to lead.

2. Experiment on lead exposure of *Chironomus* spp. and induction of mentum deformities

Before starting the experiment, chironomid larvae were cultured in concrete tube with 30 cm diameter and 40 cm height. One hundred grams of catfish feed were poured into the concrete tube, followed by 10 L of freshwater. After chironomid female laid eggs for 2 weeks, 200 second instar larvae could be harvest for the experiment in one batch. The tube continued to cultivate the larvae in order to continue the experiment until it reached 2,000 individuals for use in the 10 batches of experiment. The larvae were fed using catfish feed once a week.

In the experiment, the second instar larvae were raised in 4 different concentration of lead mixtures, including 0, 10, 20 and 30 mg/L for 10 batches. The solutions were prepared from $Pb(NO_3)_2$. At each batch, 200 second instar larvae were separated for 4 concentrations (i.e. 50 individuals per treatment) and placed into 1 L glass jar covered with lid. The larvae were feed 2 g of catfish feed per week. The experiment was conducted until the larvae grew up to the fourth instar. Then, lead solution and chironomids larvae were harvested and examined. Lead concentration in the solution and the larvae were analyzed for the residue concentration and bioaccumulation using Flame Atomic Absorption Spectrophotometer. The larvae were kept in 70% EtOH and examined under microscope. The harvested larvae were examined and identified to species and use for bioassay experiment based on Simwiset et al. (2015). All menta were examined and evaluated for the presence of deformities and labeled.

Growth rate, 50% lethal concentration (LC_{50}) and bioaccumulation factor (BAF) were calculated. LC_{50} was calculated by regression equation. While bioaccumulation factor (BAF) calculation was followed Demina et al. (2009)- i.e. concentration of lead in organisms divided by concentration of lead in water.

Results

1. A survey on mentum deformities of *Chiromomus* spp. in Huai Kakang stream

1.1 Analysis of physicochemical parameters

The main physical and chemical characteristics of the sampling station are shown in Table 1. According to the general water quality parameter, they were not in excess of the standard limits established by the National Standard Surface Water. However, the values were closed to the limits in both sites, especially COD and orthophosphorus, which indicated polluted water.

Upon analysis of the heavy metals in the water, all metals were detected, only Pb and Cd were detected at the sampling sites exceeding the limits specified as the maximum permissible established by the National Standard on Freshwater Sediment.

All the metals analyzed were detected in the sediments- i.e. Pb, Zn, Cd, Cu and Ni. The metals detected at both sites were not distinct different. Therefore, the water quality assessment indicated that Huai Kakang stream was polluted from the upper through the downstream. And the upper stream could not be called for the reference site.

Table 1 Physicochemical variables of water and sediment, average and standard deviation (SD), measured at the two sampling sites in the Huai Kakang stream

Parameters	Standard limits	Mean ± S.D.	
		Upper stream site	Lower stream site
pH in water	5.0-9.0	6.56 ± 0.09	7.54 ± 0.08
DO	Not lower than 6.0 mg/L	1.95 ± 1.70	3.53
Water temperature	-	31.05 ± 1.48	33.35 ± 0.3
COD (mg/L)	Not above 120 mg/L	111.80 ± 36.10	77.76 ± 5.40
Nitrate (mg/L) in water	Not above 0.5 mg/L	-	-
Orthophosphate (mg/L) in water	0.01-0.1 mg/l	0.51 ± 0.50	0.61 ± 0.50
Pb in water	Not above 0.05 mg/L	0.19 ± 0.01	0.19 ± 0.01
Pb in sediment	Not above 400 mg/Kg	16.8 ± 10.1	14.25 ± 5.5
Zn in water	Not above 1.00 mg/L	0.28 ± 0.03	0.25 ± 0.03
Zn in sediment	-	31.95 ± 24.40	31.50 ± 19.80
Cd in water	Not above 0.005 mg/L	0.07 ± 00	0.07 ± 00
Cd in sediment	Not above 37 mg/Kg	3.60 ± 00	3.60 ± 00
Cu in water	Not above 1.00 mg/L	0.13 ± 00	0.12 ± 00
Cu in sediment	-	12.50 ± 00	13.05 ± 7.00
Ni in water	Not above 0.1 mg/L	0.09 ± 00	0.09 ± 00
Ni in sediment	Not above 1,600 mg/Kg	7.70 ± 00	6.90 ± 2.80

1.2 *Chironomus* spp. throughout a polluted gradient

In this study the following 5 species were found in Huai kakang stream, including *Chironomus calligaster, C. circumdatus, C crassiforceps, C. javanus, C. kiiensis.* At the upper stream site, 3 species- i.e. *C. calligaster, C. crassiforceps, C. kiiensis* were found, while all 5 species were found at the lower stream sites.

Total 956 of *Chironomus* spp. larvae from Huai Kakang were counted and analyzed, and shown in table 2. *Chironomus* spp. were found at the upper stream site in higher number (i.e. 656 individuals) than the lower stream site (i.e. 300 individuals). As to mentum deformity in *Chironomus* spp., individual from the upper stream site (n = 458) had no alteration in their mount structure, while 171 individuals exhibited metum deformity (26.07%). At the lower stream site, 300 individuals collected, 106 individuals exhibited mentum abnormality (53.33%).

Table 2 Summary of the number of larvae sampling and the number and percentage of mentum deformity registered at the Huai Kakang stream

	No. of *Chironomus* spp. in the upper stream site		No. *Chironomus* spp. in the lower stream site	
	Normal	Deformities	Normal	Deformities
1st sampling	190	110	194	106
	(63.33%)	(36.67%)	(64.67%)	(53.33%)
2nd sampling	295	61	-	-
	(79.67%)	(20.33%)		
Total	485	171	194	106
	(73.93%)	(26.07%)	(64.67%)	(53.33%)

2. Experiment on lead exposure of *Chironomus* spp. and induction of mentum deformities

Two thousand larvae were used in the experiments. They were identified into 4 species, comprise with *C. circumdatus, C. kiiensis, C. crassiforceps* and *C. javanus. C. circumdatus* was the most abundant species, and also showed the highest percentage of mentum deformities (Table 4).

The chironomid mouthpart deformities were clearly induced after being exposed to lead solutions. All abnormal menta showed the same characters of deformity, such as distinct asymmetry, large gap or missing teeth. At treatment of 20 mg/L of lead concentration, the larvae had the highest percentage of deformity.

The growth of chironomids showed a decrease in population. Therefore, the death rate was calculated. The death rate of chironomids in concentration of 0, 10, 20 and 30 mg_{Pb}/L were -0.06617, -0.06911, -0.07962 and -0.11568 individual/day, respectively. It indicated that the high concentration of lead caused the larvae to reduce in number (Table 3). The 50% lethal concentration (LC_{50}) was 34.74 mg_{Pb}/L, which was calculated by regression equation shown in Fig 2. The LC_{50} value closed to the experiment of 30 mg_{Pb}/L.

Table 3 Number and percentage of Chironomids larvae exposed to the different lead concentration found dead, emerged, normal mentum and deformed mentum

Lead concentration (mg_{Pb}/L)	Dead		Emerged		Normal		Deformed		Total number (ind.)	Death rate (ind./day)
	no.	%	no.	%	no.	%	no.	%		
0	302	60.4	72	14.4	100	20.0	26	5.2	500	-0.06617
10	310	62.0	47	9.4	110	22.0	33	6.6	500	-0.06911
20	336	67.2	45	9.0	83	16.6	36	7.2	500	-0.07962
30	401	80.2	35	7.0	51	10.2	13	2.6	500	-0.11568

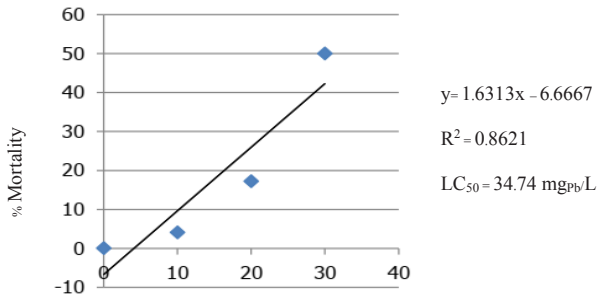

Fig. 2 Regression model on lead concentration (mg_{Pb}/L) against the percentage of mortality

The concentration of lead accumulated in the larvae was shown in table 5 and indicated that the deformed mentum larvae accumulated more high concentration than the normal larvae, especially the experiment of 20 and 30 mg_{Pb}/L. While the lower concentration (0 and 10 mg_{Pb}/L) showed less number of deformed menta larvae than the normal larvae.

The larvae in the experiment of 0 mg/L was also found the accumulated lead concentration. These may be caused by many issue such as feed and substrates used in the experiment (Mergalli et al., 2000).

Table 4 Incidences of mentum deformities among Genus Chironomus exposed to the different lead concentration

Lead concentration (mg_{Pb}/L)	Chironomid species	Normal		Deformities	
		No. of larva sampled	%	No. of larva sampled	%
0	*C.kiiensis*	14	73.68	5	26.32
	C.javanus	1	1.00	0	0
	C.cassiforceps	4	80.00	1	20.00
	C.circumdatus	81	80.20	20	19.80
	Total/average	**100**	**79.36**	**26**	**20.64**
10	*C.kiiensis*	9	81.82	2	18.18
	C.javanus	4	80.00	1	20.00
	C.cassiforceps	1	100.00	0	0
	C.circumdatus	96	76.19	30	23.81
	Total/average	**110**	**76.19**	**30**	**23.81**
20	C.kiiensis	6	66.66	3	33.34
	C.javanus	7	77.77	2	22.23
	C.cassiforceps	7	77.77	2	22.23
	C.circumdatus	63	68.48	29	31.52
	Total/average	83	69.75	36	30.25
30	C.kiiensis	4	80.00	1	20.00
	C.javanus	0	0	0	0
	C.cassiforceps	1	100.00	0	0
	C.circumdatus	46	79.31	12	20.69
	Total/average	**51**	**79.69**	**13**	**20.31**

Bioaccumulation of lead in *Chironomus* spp. larvae, which showed incidences of mentum deformities, were shown in Table 5. The experiment of 20 mg_{Pb}/L showed the highest lead concentration in the larvae of 24,863.64 mg_{Pb}/Kg wet weight/individual. The calculation result of bioaccumulation factor was 3,477.43.

Table 5 Bioaccumulation of lead in mentum deformity *Chironomus* spp. larvae and in water

Lead concentration (mg_{Pb}/L)	Bioaccumulation of Lead in Chironomids (mg_{Pb}/Kg wet weight/ individual)		Actual Lead Concentration in water (mg/L)		Bioaccumulation Factors of deformity larvae (BAF)
	Normal	Deformity	Before Experiment	After Experiment	
0	654.52	636.48	1.54	4.89	130.16
10	1420.95	657.18	8.29	5.89	117.77
20	7,964.14	24,863.64	15.75	7.15	3,477.43
30	2,435.78	4,114.13	18.08	8.67	280.94

Discussion

Many publications –e.g. Thani & Prommi (2017), Di Veroli et al. (2010), Odume et al. (2012)- indicated the relations of the presence of pollutants in freshwater habitats and mouthpart deformities. The result of this study showed the same trends of those publications in strongly supported that the deformity can be used in the bioassessment work. The screening *Chironomus* communities for the deformities in Huai Kakang stream indicated the habitat perturbation by community activities caused notable impact on water quality at all stream reach as seen by physicochemicals parameter and percentage of the deformities at the upper stream site. The percentage of deformities found in the upper stream and the lower stream sites, were 26.07% and 53.33%, respectively. This number were much higher than a deformity of Chironomids found in the freshwater ditches at Nakhon Pathom province (6-12%) (Thani & Prommi, 2017).

Many different pollutants suspected to be the majors concern of the deformities, i.e. orthophosphate (PO_4^{3-}), ammonia-nitrogen (NH_3-N), nitrate-nitrogen (NO_3^--N), dissolved oxygen (DO), pH and water temperature (Thani & Prommi, 2017), heavy metals and pesticides (Di Veroli et al., 2010). In this study, metals-especially lead and cadmium, were found exceeding the limits specified as the maximum permissible and were suspected to be a major pollutant to cause the deformities in chironomids. Consequently, we conducted the experiment on lead exposure of *Chironomus* spp. and their

mentum abnormality. On the experiment, *Chironomus circumdatus* was the common species in Maha Sarakham province, which showed the relationship with level of contaminant and the percentage of mentum deformity. We suggested using this species as a bioindicator for minoring Huai Kakhang stream water quality. Meregalli et al. (2000) said that assessing the presence of pollutants in the stream by monitoring mouthpart deformities is more suitable than using endpoints such as growth and survival. Our research showed the same results. The growth rate and LC_{50} studies indicated that the highest lead concentration caused the highest deformities of the mentum. While the percentage of deformities was found the highest percentage in the experiment of 20 mg_{Pb}/L, which the *Chironomus* spp. accumulated the highest bioconcentration of lead in the same experiment. The frequency of mentum deformity has been reported to reach 60% by Vermeulen et al. (1998), where as in the present in Huai Kakang stream was 53.33% in the impacted site, and in experiment the maximal number was 30.25% in the experiment of 20 mg_{Pb}/L. This suggested that pollution level at the Huai Kakang stream survey's sites were almost extreme. The bioaccumulation of lead concentration showed the same trend as the deformity. Again, this confirmed that biomonitoring using the mouthpart deformity of *Chironomus* spp. is more suitable. Surprisingly, Chironomids had the bioaccumulation factor (BAF) value of 3,477.43 it means a larva can accumulate lead into its body for about 3,400 times higher than water. That is why the scientist classified the Chironomidae as bioaccumulator. Another surprise was that high amount of lead accumulated in the larvae, which was much higher than the added amount. Still, we could not find a good explanation for this situation.

Conclusion

In conclusion, the stress of anthropogenic pollutions in Huai Kakang stream could be distinctly detected and may cause the problem for user in many activities, such as fishery, agriculture (normally use for rice and vegetable growing) and recreation. Therefore, the community wastewater management is urgently need for Huai Kakang stream.

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Development of Sai Krok E-san Kai (Chicken Fermented Sausages) with Riceberry Rice

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Fermented Sausages, Riceberry Rice, Consumers' Acceptance, Sai Krok E-san Kai

Abstract

This research aims to (1) study the ratios of sticky rice to Riceberry rice in Sai Krok E-san Kai with Riceberry rice, at 70:30, 50:50 and 0:100 (2) compare the nutritional value between control Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice and (3) carry out consumers' acceptance test of Sai Krok E-san Kai with Riceberry rice. The study showed that the most accepted ratio of sticky rice to Riceberry rice is 50:50, with overall liking score of 7.48±1.14 (moderately like to like very much). From the comparison of nutritional value in control Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice, it was found that the control sample had 181.29 kcal of energy, 8.29 g fat, 13.12 g carbohydrate, 2.58 g dietary fiber, 0.45 mg iron and 0.69 mg zinc. On the other hand, Sai Krok E-san Kai with Riceberry rice had 163.65 kcal of energy, 7.49 g fat, 10.34 g carbohydrate, 4.86 g dietary fiber, 0.97 mg iron and 0.75 mg zinc. Moreover, the consumer acceptance test of Sai Krok E-san Kai with Riceberry rice showed that, out of 100 panelists, 89% accepted the product and 75% decided that they would purchase the product.

Introduction

Sai Krok E-san, or fermented pork sausage, is made by packing pork meat, pork fat, cooked rice and seasonings into pork intestine, or other edible intestines, before cooking by boiling, frying, or baking. Common Sai Krok E-sans in the market are usually fermented and dried in the sunlight for only 1-2 days, making it less sour than Naem (sour pork) but safer from potentially toxic microorganisms (Thai Industrial Standards Institute, 1995).

Recently, it has been found that there are approximately 1.5 million Muslims, 55 member states, consuming over 3 trillion baht worth amount of food, of which are halal foods. Therefore, it is understandable that Muslim food entrepreneurs are keen to develop products that conform with halal standards (Halal Institute Prince of Songkhla University, 2014). In general, Halal foods are foods that can be consumed by both Muslims and non-Muslims, however observant Muslims may only consume halal foods that are defined in Islam regarding prohibited and permitted foods. According to principles in Islam, Halal foods are the foods approved by God to be consumed or utilized as they are clean, have nutritional value and are protected from uncleanliness since the selection of ingredients, production process, packaging, preservation, distribution and sale. Moreover, Muslims are also prohibited to

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consume animal blood, food derived from all toxic plants, and also any food or drink containing alcohol or hazardous ingredients (Halal Standard Institute of Thailand, 2014).

Vanichpun (2003) found the glutinous rice gave good binding of the mixed ingredients of Thai fermented sausage when compared to rice, glass noodle, mixture of rice and glass noodle and mixture of glutinous rice and glass noodle. In addition, consumers' are increasingly concerned about their health; hence, the use of Riceberry rice, which is obtained from cross-breeding Khao Jao Hom Nil and white jasmine rice no.105, is composed of high antioxidants, such as betacarotene, gamma oryzanol, vitamin E, tannin, zinc and folate; having low to medium sugar index also helps reducing the risk of cancer and lowering cholesterol levels (Akkarawinit, 2016). As a result, it is greatly interesting to use Riceberry rice as an ingredient of Sai Krok E-san Kai, as to increase the nutritional value and the suitability of being a healthy food.

As Halal food has been accepted to be hygienic, there has been an upsurge in its popularity. Therefore, the researchers would like to develop Sai Krok E-san (fermented sausage) using chicken instead of pork as a way to conform to Halal standards. Moreover, addition of Riceberry rice was studied for the optimal ratio between sticky rice and Riceberry rice, in order to develop into a healthy Halal food, so as to add an alternative product for Muslim consumers and any consumers.

The objectives of this research are study of ratio of sticky rice and Riceberry rice in Sai Krok E-san Kai and to compare the nutritional values between control Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry as well as to test for consumer's acceptance of Sai Krok E-san Kai with Riceberry rice.

Materials and methods

Materials

Chicken breast fillet was produced by Betagro Pub Co., Ltd. Riceberry rice was produced by Chia Meng Co., Ltd. (Hongthong brand). Sticky rice was produced by Sandee Rice (Thailand) Co., Ltd.

Methods

1. Study the ratios of sticky rice to Riceberry rice in Sai Krok E-san Kai with Riceberry rice

This research used chicken as a pork substitute in Standard Sai Krok E-san (Chaisorn, 2009) as shown in Fig. 1 and Riceberry rice as a sticky rice substitute, with sticky rice: Riceberry rice ratio of 70:30, 50:50 and 0:100. The Sai Krok E-san Kai with various ratios were tested for consumers' liking test with 50 panelists using 9-point hedonic scale (1 = "dislike extremely", 9 = "like extremely"); the tested attributes consisted of appearance, color, aroma, taste, texture and overall liking. The samples were coded with three-digit randomized numbers and served in sequential monadic order (Lawless & Heyman, 1998).

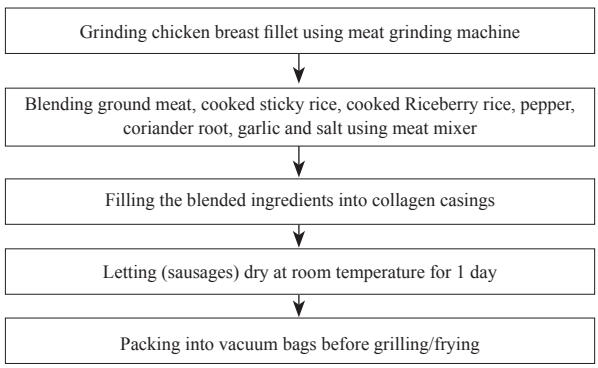

Fig. 1 Fermented sausage processing

2. Comparison of nutritional values between control Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice

Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice which had the highest score from the previous experiment were evaluated in nutritional values, chemical qualities and Microbiological qualities as follows:

Nutritional values and chemical qualities (AOAC., 2012)

1) Total Fat
2) Total carbohydrate
3) Total dietary fiber
4) Ash
5) Vitamin B1
6) Vitamin B2
7) Sodium
8) Iron
9) Zinc
10) Total Polyphenol
11) Folate

Microbiological qualities

1) Aerobic plate count
2) Lactic acid bacteria at 30°C

3. Acceptance test of consumers for Sai Krok E-san Kai with Riceberry rice

A survey for acceptance test of target consumers, consisting of 50 Muslim panelists and 50 non-Muslim panelists, that consume Sai Krok E-san Kai with Riceberry rice at least twice per month using a questionnaire, including demographic data, data of consumers' liking scores toward the product using the 9-point hedonic scale, and data of 100 panelists' acceptance using the binomial (yes/no) scale.

4. Statistical analysis

Data from formulation and nutritional values were subjected to analyses of variance (ANOVA) and t-test. The results of acceptance test were calculated with the frequency (percentage). Statistical difference was established at $p<0.05$.

Results and Discussion

1. The optimal ratio between sticky rice and Riceberry rice in Sai Krok E-san Kai with Riceberry rice

The study of ratios between sticky rice and Riceberry rice in Sai Krok E-san Kai consists of 3 ratios, i.e. 70:30, 50:50 and 0:100. The samples were tested for liking scores with 50 panelists, using 9-point hedonic scale; the results in each attribute are as follows (Table 1).

In visual aspect, the ratio between sticky rice and Riceberry rice of 70:30 and 50:50 had the highest liking score of 7.63 – 7.65 (like moderately to like very much). The liking scores of 0:100 sticky rice to Riceberry rice ratio were averaged to be 6.73 (like slightly to like moderately).

With respect to colors, the ratio of 70:30 sticky rice to Riceberry rice had the highest liking score of 7.51 (like moderately to like very much), the 50:50 ratio had an average liking score of 7.38 (like moderately), and the 0:100 ratio had an average liking score of 6.56 (like slightly to like moderately). Due to the red-violet color of Riceberry rice (Akkarawinit, 2016), the Sai Krok E-san therefore had a dark color, unusual from common Sai Krok E-san.

For aroma, the 50:50 ratio of sticky rice: Riceberry rice had the highest liking score of 7.55 - 7.56 (like moderately to like very much), and the 0:100 ratio of sticky rice: Riceberry rice had an average score of 6.60 (like slightly to like moderately).

Regarding taste, the 50:50 and 70:30 ratios of sticky rice to Riceberry rice had the highest liking score of 7.21 - 7.23 (like moderately), while the 0:100 ratio of sticky rice to Riceberry rice had an average score of 6.55 (like slightly to like moderately).

For the texture, the 50:50 ratio of sticky rice to Riceberry rice had the highest liking score of 7.31 (like moderately). The 70:30 ratio of sticky rice to Riceberry rice had an average score of 7.10 like moderately) and the 0:100 ratio of sticky rice to Riceberry rice had an average score of 6.50 (like slightly to like moderately).

In overall liking, the 50:50 ratio of sticky rice to Riceberry rice had the highest liking score of 7.58 (like moderately to like very much). The 70:30 and 0:100 ratios of sticky rice to Riceberry rice had average scores of 7.35 (like moderately) and 6.46 (like slightly to like moderately), respectively.

Considering the results, it can be concluded that the 50:50 ratio of sticky rice to Riceberry rice was the most appropriate for producing Sai Krok E-san Kai with Riceberry rice, as it had the highest liking scores in various attributes, i.e. appearance, aroma, taste, texture and overall liking. Furthermore, the product is able to have up to 50% of Riceberry rice as sticky rice substitute, increasing the nutritional benefits from Riceberry rice; henceforth, making the development of healthy product suitable.

Table 1 Average liking scores of Sai Krok E-san Kai with different Riceberry rice proportions

Sensory characteristics	Sticky rice : Riceberry rice ratio 70 : 30	Sticky rice : Riceberry rice ratio 50 : 50	Sticky rice : Riceberry rice ratio 0 : 100
Appearance	7.65 ± 1.02^{a}	7.63 ± 0.99^{a}	6.73 ± 1.07^{b}
Color	7.51 ± 1.49^{a}	7.38 ± 0.92^{b}	6.56 ± 1.03^{c}
Aroma	7.55 ± 1.15^{a}	7.56 ± 1.12^{a}	6.60 ± 1.19^{b}
Taste	7.21 ± 1.24^{a}	7.23 ± 1.30^{a}	6.55 ± 1.09^{b}
Texture	7.10 ± 1.27^{b}	7.31 ± 1.05^{a}	6.50 ± 1.04^{c}
Overall liking	7.35 ± 1.17^{b}	7.58 ± 1.14^{a}	6.46 ± 1.12^{c}

Remark: Means in rows followed by different letters represent significant differences ($p<0.05$).

2. Nutritional values and chemical qualities of Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice

According to Table 2, the comparison of nutritional values and chemical qualities between that of Sai Krok E-san Kai and that of Sai Krok E-san Kai with Riceberry rice showed that the developed Sai Krok E-san Kai with Riceberry rice had lower energy and higher total dietary fiber, iron and zinc than standard Sai Krok E-san Kai, while having less total fat and total carbohydrate. Therefore, the developed Sai Krok E-san Kai with Riceberry rice is a suitable alternative of healthy products.

Table 2 Nutritional values and chemical qualities of Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice (per 100 g)

Nutritional values	Sai Krok E-san Kai	Sai Krok E-san Kai with Riceberry rice
Energy (kcal)	181.29 ± 0.12[a]	163.65 ± 0.10[b]
Moisture (g)[ns]	62.47 ± 0.02	65.84 ± 0.02
Protein (g)[ns]	13.55 ± 0.08	13.72 ± 0.06
Total Fat (g)	8.29 ± 0.02[a]	7.49 ± 0.03[b]
Total Carbohydrate (g)	13.12 ± 0.09[a]	10.34 ± 0.05[b]
Total dietary fiber (g)	2.58 ± 0.01[b]	4.86 ± 0.02[a]
Soluble dietary fiber (g)	0.16 ± 0.00[b]	0.20 ± 0.00[a]
Insoluble dietary fiber (g)	2.42 ± 0.00[b]	4.66 ± 0.00[a]
Ash (g)[ns]	2.57 ± 0.02	2.61 ± 0.01
Vitamin B1 (mg)[ns]	0.06 ± 0.00	0.07 ± 0.00
Vitamin B2 (mg)[ns]	0.10 ± 0.00	0.12 ± 0.00
Sodium (mg)	823.08 ± 0.10[a]	808.84 ± 0.10[b]
Iron (mg)	0.45 ± 0.00[b]	0.97 ± 0.00[a]
Zinc (mg)	0.69 ± 0.00[b]	0.75 ± 0.00[a]
Total polyphenol (mg ea GA)[ns]	56.73 ± 0.10	57.52 ± 0.10
Folate (mcg)[ns]	18.00 ± 0.01	18.00 ± 0.01

Remark: Means in rows followed by different letters represent significant differences ($p<0.05$)
[ns] = Means in row are not significant differences ($p>0.05$).

According to pricings, Sai Krok E-san Kai costs 38.2 baht, and Sai Krok E-san Kai with Riceberry rice costs 38.5 baht. While their costs are somewhat similar, Sai Krok E-san Kai with Riceberry rice contains more insulation fiber. Among numerous health benefits of fiber, a high-fiber diet is associated with a lower risk of many disease, including obesity, cardiovascular disease, diabetes, metabolic syndrome and others. (Lattimer & Haub, 2010)

From Table 3, the results from microbial test in Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry rice (per 100 g) showed that Sai Krok E-san Kai had an aerobic plate count of 8.4 x 10^8 CFU/g, and lactic acid bacteria of 1.9 x 10^8 CFU/g, while Sai Krok E-san Kai with Riceberry rice had an aerobic plate count of 1.2 x 10^9 CFU/g, and lactic acid bacteria of 1.1 x 10^8 CFU/g, which conforms to Thai Industrial Standards Institute (1995) and Thai Community Product Standard (2003) about "Sai Krok E-san".

Table 3 Results of microbial test in Sai Krok E-san Kai and Sai Krok E-san Kai with Riceberry (per 100 g)

Microbiological qualities	Sai Krok E-san Kai	Sai Krok E-san Kai with Riceberry rice
Aerobic plate count (CFU/g)	8.4 x 10^8	1.2 x 10^9
Lactic acid bacteria at 30 °C (CFU/g)	1.9 x 10^8	1.1 x 10^8

4. Acceptability test

The respondents of the questionnaire for the study of consumers' acceptance for Sai Krog E-san Kai with Riceberry rice were 74% female and 26% male. 52% of them were aged between 41 – 60 years of age. 36% were between 20 – 40, while 9% were the elders. In terms of income, 38% of the respondents earned 15,000-30,000 Baht per month. 32% earned monthly less than 15,000 Baht, while 32% had no income.

Most respondents pointed out they consumed Sai Krog E-san Kai when they felt like it (53%). 41% reported to have Sai Krog E-san Kai during a trip and 40% during different activities, be they reading or watching TV. The reasons for consuming Sai Krog E-san Kai included desirable aroma and taste (89%), convenience of consumption (40%), reasonable price for the quality (36%). The frequency of consumption entailed once a week (54%), 2-3 times per week (19%) and once a month (13%).

92% of the respondents pointed out that they are interested in the product. Most respondents (89%) accepted Sai Krog E-san Kai with Riceberry rice. 75% of the respondents showed interest in purchasing Sai Krog E-san Kai with Riceberry rice. All were presented in Table 4.

Table 4 Consumer acceptance of Sai Krog E-san Kai with Riceberry rice

Data items	Percentage
Gender	
Male	26
Female	74
Age	
Below 20	3
20-40	36
41-60	52
Above 61	9
Income	
No income	20
Below 15,000 Baht	32
15,001-30,000 Baht	38
30,001-50,000 Baht	10
Above 50,000 Baht	0
Time of consumption	
Feeling like	53
As a snack with drinks	27
During a trip	41
During activities, e.g. reading or watching TV	40
Others	2
Reasons for consumption	
Olfactory and gustatory sensation	89
Right pricing for quality	36
Convenience in consumption	40
Nutritional value	20
Others	1

Table 4 Consumer acceptance of Sai Krog E-san Kai with Riceberry rice (Cont.)

Data items	Percentage
Frequency of consumption	
Everyday	0
3-4 times/week	5
2-3 times/week	9
1 time/week	19
1 times/month	54
Others	13
Interest in healthy E-san chicken sausage	
Interested	92
Not interested	8
Do you accept riceberry E-san chicken sausage?	
Yes	89
No	11
Would you buy riceberry E-san chicken sausage, once it is on sale	
Yes	75
No	25

Conclusion

The results from developing Sai Krok E-san Kai with Riceberry rice showed that the optimal ratio of sticky rice to Riceberry rice is 50:50, which led to the utilization of Riceberry rice which has high antioxidant, but provides low energy, fat, and carbohydrate. It also has other nutritional values such as dietary fiber, iron and zinc, higher than those in standard Sai Krok E-san with 100% sticky rice. The Sai Krok E-san with Riceberry rice product has microbial quality conforming to the regulations. Results of consumers' acceptance test showed that 89% of consumers accepted the developed product and 75% would purchase the product if available in the market. Therefore, Sai Krok E-san with Riceberry rice can be a new product that would respond to the demands from all consumers including Muslim consumers and consumers who are concerned about the health.

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Bioremediation of Persistent Organic Pollutants in Environment: Alternatives and Limitations

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Abstract

The contamination of persistent organic pollutants (POPs) in environment has been increasing in recent years due to anthropogenic activities. The toxicity of POPs even in the concentration at nanogram levels make them a concern for harmful effects on human health and environment. Human and animal exposure to POPs can lead to various effects ranging from skin and eye irritation, liver and kidney toxicity, nervous system damage, to cancer and death. So far, proper techniques for remediation of POPs are still unresolved. The chemical treatment methods are considered not environmental-friendly and cost-effective; therefore, remediation has favorably turned to biological techniques. Natural microorganisms have been shown by many studies to be able to degrade hazardous pollutants; however, the direct use of only environmentally-isolated microorganisms does not always result in effective remediation of POPs due to their toxic and recalcitrant nature. Therefore, effective strategies should be considered before applying the techniques for bioremediation of POPs. By examining the most recent research and studies for biodegradation of POPs, this review aims to describe the alternatives of bioremediation of POPs, which are the implementation of specific or adapted strains, the application of plant-microorganism interconnected relationships, and the utilization of enzymes. Moreover, the factors that can limit the complete bioremediation of POPs are also discussed. The gaps for improvement provided by previous studies should be able to pave the way for further studies to develop new techniques for bioremediation of POPs in contaminated sites.

Introduction

In addition to the increasing industrial and agricultural activities, with the advancement of electronics, pharmaceuticals, and medicines, more organic chemicals have been newly synthesized for human uses. Some of these chemicals showed the evidence of high persistence, potential bio-accumulation and adverse effect on human health or the environment, and also the potential for long-range transport; These compounds or persistent organic pollutants (POPs) have been a contamination problem for decades. In 2001, the

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Stockholm Convention on POPs listed twelve compounds as the initial POPs; as of 2017, the list is now expanded to cover sixteen more compounds (Table 1). These POPs can be categorized into three groups, e.g., pesticides, industrial compounds and unintentional by-products depending on the source information or use purposes. When released into the environment, POPs are considered as the emerging pollutants since their contamination is new to our generation and quite unknown for the methods of remediation. The toxicity of POPs, ranging from skin irritation to nervous damage or cancer, makes them harmful not only for human but also environment even in only small concentrations. Studies showed that these POPs can stay in environment for a very long time and hard to be degraded by light and oxygen alone (Arslan et al., 2017).

Various technologies have been proposed for the treatment of POPs. Some of the effective treatment methods such as advanced oxidation processes seem to rely on the ex-situ treatment where the portion of contaminated soil or water is removed from the field. These techniques require high capital cost and need an expert for maintenance. Inexpensive remediation techniques where they can be applied in-situ are preferable.

Bioremediation is a remediation technique that can be operated with a low-cost investment. A very successful bioremediation is commonly achieved in laboratory conditions; however, application of bioremediation needs to overcome some challenges on diverse environmental conditions that can affect not only the microorganisms but also the conditions for degradation of POPs.

This review is divided into 3 sections. First, the contamination and effects of POPs are given out to emphasize on the severe effects of them on environment and why they requires treatment. Next, the options for applying bioremediation in the contaminated sites are presented. Since there are many uncertainties in environment that can hinder the successful bioremediation, the factors limiting the bioremediation of POPs will be described in the last section.

Contamination and effects of POPs

Besides agricultural and industrial uses, POPs can be found from the residues of everyday products including cosmetics, disinfectant, antibiotics, anti-inflammatories, wood preservatives, paint additives, antidepressants, plasticizers and phthalates (Tripathi et al., 2015). There have been many reports on the contamination of POPs in soil and water ecosystems. It was found that wind speed, seasonal change, temperature variation, and land-use activities play an important role for environmental contamination and effects of POPs (Alharbi et al., 2018). The concentrations of conventional POPs such as PCBs, DDT, and some PAHs were found in soil ranging from 2 ng up to 7 µg (Zhu et al., 2014). Halogenated POPs such as trichloroethylene (TCE) have been a concern for over a decade due to the prevalence in soil and water. Since the POPs are hydrophobic in nature, they tend to sorb with the organic partitions, which are organic matter in soil particles and organic constituents in water and sediment (Ren et al., 2018b). The problem of POPs in Asia is projected to worsen according to the environmental standards that have been set to the higher levels than the guidelines set by the US EPA and EU. For example, the amount of organochlorine compounds as hexachlorocyclohexane (HCHs) is recommended below 950 ng/L by the US EPA's National Recommended Water Quality Criteria for aquatic life and is at 500 ng/L for the EU Drinking Water Regulations while the China environmental quality standards for surface water limits at 2,000 ng/L (Han & Currell, 2017). In Thailand, according to the Pollution Control Department, the national surface water quality standards for the organochlorine compounds are set at 5×107 ng/L for total organochlorine pesticides and at 20,000 ng/L for Heptachlor and Heptachlorepoxide (source: www.pcd.go.th).

POPs have been reported to generate oxidative stress, which creates an inflammatory response in the cellular level (Petriello et al., 2014). Organisms such as fish and insects exposed to POPs can result in birth defects and abnormalities (Chakraborty & Das, 2016). Also, it was reported that POPs can accumulate not only in animals but also in plants (Zhu et al., 2014). In case of humans, because POPs are highly soluble in lipid, the accumulation in human tissue is likely to happen. This leads to inflammatory diseases and the increased risk of chronic diseases (Petriello et al., 2014). Furthermore, many of POPs are known to be carcinogenic and mutagenic (Zhu et al., 2014). The stability of POPs in environment is high, and it will be longer for the higher chlorinated compounds (Arslan et al., 2017).

Alternatives for bioremediation of POPs

The chemical treatment techniques like advanced oxidation processes (AOPs) have been popularly used

against the recalcitrant organic pollutants in soil and water. Since the structure of POPs is hard to break down, the most effective techniques rely on the generation of hydroxyl radicals (•OH), which is a very powerful oxidant, to break or destroy the specific chemical bonds in the target organic pollutants. It has been proved that various AOPs such as ozonation, Fenton oxidation, or photocatalysis were able to remove both conventional POPs such as organochlorine insecticides, solvents, and polychlorinated biphenyls and emerging POPs such as antibiotics, hormones, and drugs from water and wastewater (Ikehata et al., 2008). However, the generated hydroxyl radicals attack compounds non-specifically, which means not only the targeted POPs but also the surrounding organic matters; The excavation of contaminated soil or water, and also the extraction of POPs are required. Accordingly, an operation and maintenance cost can be the drawback when using the chemical or physicochemical treatments. Also, not many studies have been conducted on the mineralization and the bioavailability of the metabolites and degradation products after using AOPs (Ikehata et al., 2008). Furthermore, remediation techniques using chemicals will be considered 'not green' for environment. Even though some studies have successfully used environmentally friendly biopolymer for adsorption of POPs (Pariatamby & Kee, 2016), further management of the biopolymer waste containing POPs is required.

Microorganisms are the major group of living organisms in the environment, existing as free-living cells or biofilm. They play roles in many natural/ environmental processes including bioremediation. Due to their high diversity and adaptability, microorganisms show great potential for POPs degradation. The successful POPs degradation by bacteria, fungi, and yeasts have been reported (Oyetibo et al., 2017). POPs can be degraded by being a direct substrate/carbon for microbial growth or getting destroyed through other biochemical pathways in a co-metabolism process (Oyetibo et al., 2017). POPs can be transformed by chemoorganotrophs through several pathways; for example, oxidation, reduction, hydrolysis and dehalogenation under both aerobic and anaerobic conditions (Chakraborty & Das, 2016; Ewald et al., 2019; Tripathi et al., 2015; Watanabe & Yoshikawa, 2008). The dehalorespiration of POPs by anaerobes yield the ATP and also the less halogenated congeners which are likely less toxic (Jeon et al., 2016; Sharma et al., 2018). However, the aerobic degradation of POPs appears to be a more effective alternative for POPs removal due to higher respiration-mediated energy yield since it could support microbial growth and POPs transformation (Jeon et al., 2016). Beside free-living cells, biofilms, the form of microorganisms living as a community within the extracellular polymers, have been found effective to degrade POPs as they have their biofilm structure to tolerate toxicity and create suitable conditions for growth (Gaur et al., 2018).

To ensure that biodegradation works successfully in the field, the survivability of the working bacteria in the environment, which needs to tolerate not only the toxicity from POPs but also harsh environmental conditions must be taken into account. Methanotrophs, for example, are one group of bacteria that can be isolated in a wide variety of habitats that can oxidize target pollutants including POPs by using the wide-range methane monooxygenase enzymes (Pandey et al., 2014). However, more research on using this group of bacteria is still ongoing whether they can adapt to various stresses in the field including pH, temperature, salinity, drought, and different chemicals (Jiang et al., 2010). Research has developed various methods in order to successfully apply bioremediation, which can be summarized as the possible alternatives for bioremediation of POPs shown in Figure 2. It should be noted that the ex-situ treatments such as bioreactor or slurry reactor are not discussed in this review. This is because despite having high removal rate in a short treatment time, ex-situ treatment requires the excavation or removal of soil and water, which increases the cost of treatment and needs specific adjustment varying among the contaminated sites (Eibes et al., 2015).

The genetic modification is also a potent approach for enhancing the efficiency of bioremediation (Singh et al., 2011). The biodegradation does not only depend on whether the microbes can produce degradative enzymes but they also need to endure the unfavorable conditions and grow fast enough to compete with other native microbes. Accordingly, unlike the laboratory or other foreign strains, the indigenous microbes appears to be a better choice for genetic engineering, e.g., introduction of the degradative enzyme, due to their higher chance of survival in the local environment (Singh et al., 2011). To construct a strain of genetically engineered microbes, the involved enyzyme(s), mechanisms or pathway need to be well laid. For examples, the isolate *Rhodococcus* sp. strain p52, is able to degrade wide ranges of contaminants including dioxins owing to two dioxygenases encoded by *dbfA* and *dfdA* (Peng et al., 2012). These two

dioxygenases are involved in the important ring dihydroxylation during dioxin degradation. The two genes are located on the two different self-transmissible plasmids of the strain p52 thus they can be transferred to other bacteria including *B. cereus* (Peng et al., 2012), *Pseudomonas aeruginosa* and activated sludge bacteria (Ren et al., 2018a; Sun et al., 2017). After mated with the strain p52, the activated slude bacteria showed the almost complete degradation of 300 mg/L dibenzofuran, a model compound for dioxin degradation, within 50 hours whereas only 50 and 150 mg/L of dibenzofuran were degraded by the unmated activated sludge bacteria and the strain p52, respective (Sun et al., 2017). After 96 days, the activated sludge in the laboratory-scale sequenctial batch reactor (SBR) bioaugmented with strain p52 could completely degrade dibenzofuran whereas only 53% of dibenzofuran was removed in the nonbioaugmented SBR (Ren et al., 2018a).

Instead of using the whole-cell biocatalyst for biodegradation, applying only the isolated enzymes provides many advantages over the whole cells including no requirement for in-situ growth, easier handling and storage, and the comparable activity for biodegradation compared with cells (Eibes et al., 2015). Furthermore, using enzymes is less stringent than using the genetically engineered cells in many countries. Enzymes can be applied as a free form or immobilized with media, which is to prevent the loss of enzymatic activity. Normally in environment, enzymes tend to bind with the mineral and organic part of soil particles (Zimmerman & Ahn, 2011); however, the enzyme-organo-mineral relationships and interactions that contribute to the biodegradation of POPs still need to be elucidated.

Plants also play roles in POPs removal via several mechanisms. Some POPs, for example, PCBs can be adsorbed to plant root (phytostabilization/rhizofiltration) or taken up into plant tissue (phytoextraction) then volatilized into the atmosphere (phytovolatilization) or transformed (phytotransformation) (Aken et al., 2010). The typical pathway for phytotransformation of xenobiotics is as shown in Fig. 1. Plants appear to be a promising alternative for POPs remediation; however, its slow remediation rate is still a major challenge and that the accumulation of POPs may be toxic to plant cells. The use of plants and certain microorganisms has been found useful and could accelerate the remediation of POPs through their symbiotic relationship. Plants and their associated bacteria include ryegrass with *Pseudomonas* sp. and *Rhodococcus* sp.; Italian ryegrass, burdsfoot trefoil, and alfalfa with *Enterobacter ludwigii;* and corn and wheat with *Burkholderia cepacia* (Andria et al., 2009; Wang et al., 2010; Yousaf et al., 2011). Microorganisms can either reside inside plant tissue (endophytic microorganisms) or localize around plant roots (rhizospheric microorganisms). While plants provide nutrients and space for microbial colonization (Nanasato & Tabei, 2018; Zhu et al., 2014), these microorganisms, in return, could promote plant growth, help plant tolerate abiotic stress, preventing plant pathogen and degrade the xenobiotics including POPs in soil/water (Chakraborty & Das, 2016; Dimkpa et al., 2009; McGuinness & Dowling, 2009). Besides acting as a carbon and energy sources for rhizospheric microbes, root exudates can also act as an inducer for some enzymes in microbial degradative pathways (Jha et al., 2015). Growing the rhizospheric *Rhodococcus erythropolis* in the presence of a non-carbon source flavanone, which is a major component in root exudate of *Arabisopsis thaliana,* together with sodium acetate could enhance the degradation of 4-chlorobiphenyl (Toussaint et al., 2011). Pham et al. (2015) further reported the similar effect of other plant flavonoids on 4-chlorobiphenyl degradation and the 13-fold up-regulation of *bphA* (encoding for large subunit of biphenyl 2,3-dioxygenase) induced by isoflavone (Pham et al., 2015). Microbial-mediated process is the mojar POPs removal in the environment (Zhu et al., 2014). In addition to the plant and microbe-assisted remediation, the transgenic plants containing microbial catabolic enzymes have also been studied for POPs removal (Rylott et al., 2015). This plant-based remediation technologies can also provide products for further uses such as biofuels, biomass, and the chemicals extracted from the biomass (Tripathi et al., 2015).

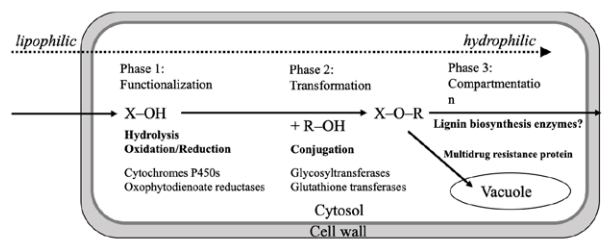

Fig. 1 The typical transformation/degradation pathway of xenobiotics in plant cell (Rylott et al., 2015).

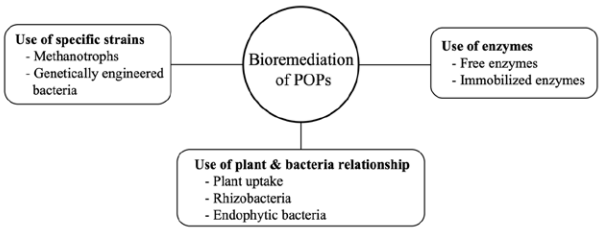

Fig. 2 Possible alternatives for bioremediation of POPs.

Factors limiting bioremediation of POPs

Generally, bioremediation of POPs can be applied using two methods: bioaugmentation and biostimulation. These two different techniques require different considerations. If non-native microorganisms are introduced to environment (bioaugmentation), the survivability of them in the field needs to be ensured; however, if the native species are induced for biodegradation (biostimulation), the condition adjustment is required. Either way, it is clear that environmental conditions and parameters are crucial for bioremediation of POPs. This is because environmental bioremediation of POPs by microorganisms depend on the conditions to allow microbial growth and metabolism of POPs (Pariatamby & Kee, 2016). It is difficult to achieve the same conditions for bioremediation in the contaminated sites as in laboratories, and the results are usually different between the lab-scale and pilot-scale treatments (Varjani et al., 2017).

Although the indigenous bacteria can be genetically engineered to increase the ability to withstand harsh conditions or to effectively degrade POPs (Chakraborty & Das, 2016), it is not always the case that the genetically engineered bacteria will be able to survive and work for a long period of time. Also, low public acceptability for using genetically engineered organisms can reduce the chance of in-situ application despite their high degradation efficiency (Singh et al., 2011). Even though enzymes are more easy-to-use than whole bacterial cells, the low stability of certain enzymes still needs to be improved. Studies have shown that immobilization of enzymes on nanoclay, metal minerals, and organic acids, which serve as the enzyme carriers, might be required in order to protect the denaturation of enzymes and ensure the effectiveness of POP degradation (Eibes et al., 2015).

In the presence of too many types of POPs, bioremediation using plants and microorganisms does not likely to give the desired degradation. Also, climate conditions are likely to affect the interactions between plant and microbes as well as the fate and transport of pollutants, all of which result in different degree of bioremediation (Tripathi et al., 2015). Factors affecting bioremediation of POPs by plants and plant-microbe interactions are bioavailability of POPs to bacteria, plant and bacteria tolerance to the toxicity of POPs, and the contribution of each bacteria survivability and detoxification ability to the whole plant-microbe community (Arslan et al., 2017).

Lastly, despite being the inexpensive and environmentally friendly techniques, bioremediation still has a challenge to overcome the treatment time (Ashraf, 2017). The biological treatment has been proven efficient for removal of hazardous substances, but more research should focus on developing a process that is less time-consuming as well.

Conclusion

From the cost and effectiveness point-of-view, bioremediation is among the most preferable techniques for remediation of POPs. Even though it seems promising, it is not a quick tool to use since it needs optimization. The options for bioremediation of POPs do not limit to building the treatment system or reactor but include bioaugmentation and biostimulation. Therefore, research in this area is still required to develop a stable system that can be applicable in the field. Using the native soil and aquatic bacteria will eliminate the problems of losing the specific species to the indigenous species. In summary, the isolation of indigenous microorganisms capable of degrading these emerging pollutants should still be going on along with the novel methods to control the environmental parameters important to biodegradation during the application of bioremediation.

Table 1 Initial and new POPs with their sources and health risks

Compounds	Sources	Health risks	Annex
Initial 12 POPs			
Aldrin	Pesticide	Nervous system and kidney effects	A: Elimination
Chlordane	Pesticide	Nervous system effects	A: Elimination
DDT	Pesticide	Liver and reproduction effects	B: Restriction
Dieldrin	Pesticide	Nervous system and kidney effects	A: Elimination
Endrin	Pesticide	Central nervous system effects on high dose	A: Elimination
Heptachlor	Pesticide	Effects on liver and fertility	A: Elimination
Hexachlorobenzene	Industrial compound	Nervous system and liver effects	A: Elimination
Mirex	Pesticide	Effects on eyes, thyroid, nervous and reproductive systems	A: Elimination
Toxaphene	Pesticide	Liver effects	A: Elimination
PCBs	Industrial compound	Skin and liver effects, probable human carcinogens	A: Elimination
Polychlorinated dibenzo-p-dioxins	Unintentional by-product	Liver, gastrointestinal, and endocrine system effects	C: Reduce the unintentional release
Polychlorinated dibenzofurans	Unintentional by-product effects	Liver, gastrointestinal, and endocrine system	C: Reduce the unintentional release
The new POPs (according to the Stockholm Convention)			
Alpha hexachlorocyclohexane	Pesticide and unintentional by-product of lindane	Potential carcinogen	A: Elimination
Beta hexachlorocyclohexane	Pesticide and unintentional by-product of lindane	Potential carcinogen	A: Elimination
Chlordecone	Pesticide	Effects on liver, nervous, and reproductive systems	A: Elimination
Decabromodiphenyl ether (commercial mixture, c-decaBDE)	Industrial compound	Endocrine toxic effects	A: Elimination
Hexabromobiphenyl	Industrial compound	Endocrine and reproductive system effects	A: Elimination
Hexabromocyclododecane	Industrial compound	Potential effects of respiratory and gastrointestinal systems	A: Elimination
Hexabromodiphenyl ether and heptabromodiphenyl ether (commercial octabromodiphenyl ether)	Industrial compound	Neurotoxic effects	A: Elimination
Hexachlorobutadiene	Industrial compound, unintentional by-product	Effects on kidney and liver	A: Elimination, and C: Reduce the unintentional release
Lindane	Pesticide	Moderately toxic and effects on nervous system	A: Elimination
Pentachlorobenzene	Pesticide, industrial compound, unintentional by-product	Central nervous system effect	A: Elimination, and C: Reduce the unintentional release
Pentachlorophenol and its salts and esters	Pesticides	Effects on liver, blood and nervous systems	A: Elimination
Perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride	Industrial compound	Effects onthyroid function and fertility	B: Restriction
Polychlorinated naphthalenes	Industrial compound, Unintentional by-product	Skin and liver effects	A: Elimination, and C: Reduce the unintentional release
Short-chain chlorinated paraffins (SCCPs)	Industrial compound	Skin and developmental effects	A: Elimination
Technical endosulfan and its related isomers	Pesticides	Respiratory and skin effects	A: Elimination
Tetrabromodiphenyl ether and pentabromodiphenyl ether (com-mercial pentabromodiphenyl ether)	Industrial compound	Neurotoxic effects	A: Elimination

Sources: Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services (2019); United States Environmental Protection Agency (2009)

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Book Review

Panaya Kotchaplai

Book name:	Industrial Biorefineries and White Biotechnology
Authors:	Pandey, A., Höfer, R., Taherzadeh, M., Nampoothiri, K.M., & Larroche, C.
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Language:	English
ISBN:	978-0-444-63453-5

"Industrial Biorefineries and White Biotechnology" is a collection of the informative chapters that gives an overview, discussion and exemplifies the application of biotechnology from the industrial standpoint. As can be presumed by the title, this book covers two main topics, the industrial biorefineries, a concept of converting industrial-based biomass to more valuable products, and white biotechnology or the industrial biotechnology.

The first part, industrial biorefineries, focuses on the biorefinery of various products, by-products or waste from industrial processes. Covering not only the definition, principle, and comprehensive overview, but also the SWOT analysis, the first chapter provides such an excellent introduction to biorefinery concept. Background, state-of-art knowledge and technologies of several key industrial-based biomass biorefinery, for example, lignocellulose, oil and algae are discussed in individual chapters. In the second part, white biotechnology, the reader will go through the detailed information regarding the development, production and application of biocatalysts, bioprocesses and bioproducts. The covered topic ranges from food, cosmetics, biosurfactant to bioplastic application. Several chapters cover the development of biocatalyst e.g. metabolic engineering for the specific industries.

Altogether, "Industrial Biorefineries and White Biotechnology" helps reader not only understand the general aspect but also provides the profound knowledge of biotechnological application in different industries. This book is recommended for students, academic, researcher and practitioners.

Reviewer

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5. The qualitative assessments of the expert panel returned by the manuscript's author. The author is expected to make the appropriate alterations indicated by the experts' feedback.
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3. The manuscript's content is consistent with the aim and scope of the journal.

4. Manuscripts submitted to Journal of Food Health and Bioenvironmental Science must not have been published previously in or actively involved in the publication process of another journal.

5. All content within the manuscript must be the product of the author himself. Any use of intellectual property within must be appropriately credited to its original authors.

6. The author must comply with the writing style established by Journal of Food Health and Bioenvironmental Science.

7. There are four levels of assessments given to reviewed manuscripts:

7.1 Requires minor or no revisions prior to publication.

7.2 Requires moderate revisions prior to publication.

7.3 Requires intensive editing and revisions followed by a future evaluation. 7.4 Unsuitable for publication

In order to be assigned the "Accepted" status, an article must be assessed as “Requires minor or no modification prior to publication” by two of the three experts from the peer review process.

Formatting Guidelines

It is the author's responsibility to format manuscripts to the standards of Journal of Food Health and Bioenvironmental Science. The details of format style are contained herein,

1. Format

1.1 Single page printing on A4 paper with a width of 19 cm and height of 26.5 cm. The vertical and horizontal spacing from the margins must be 3.5 cm and 2.5 cm, respectively.

1.2 Typefaces and layout: English must be typed using Time New Roman using Microsoft word. Specific font format guidelines are as follows.

1.2.1 The header contains the page number, aligned on the right side, in 12 pt. font.

1.2.2 The title in English languages must be 12 pt. font, bolded, and center aligned. The title should not exceed two lines of text.

1.2.3 The author's name in English language must be typed 9.5 pt. font and centered below the title. Asterisks (*) should proceed the authors' names which is correspond to the appropriate author.

1.2.4 Affiliations should match each author with their appropriate affiliated institutions and organizations. In case of different affiliations, superscript numbers should follow the surname a and affiliation a.

1.2.5 A footnote must be placed on the first page of the article with the text “*Corresponding Author”, and the next line of text should contain “e-mail”.

1.2.6 “Abstract” in English must be 9.5 pt. font, bolded, left aligned, and placed below the Thai keywords section. Abstract text must be 9 pt. font, with 1 tab indentation from left and right margins.

1.2.7 “Keywords:” should appear in English language in 9.5 pt. font, placed beneath the English abstract text and be aligned with the left margin. English keywords must be 9 pt. font, and should not exceed four words. Each keyword should be separated by a comma (,) and space.

1.2.8 Regardless of language choice, the main text headings used throughout the paper must be 9.5 pt. font, bolded, and aligned with the left margin.

1.2.9 Bulleted items must appear as 9 pt. font, bolded, and be indented 1.5 tabs from the left margin.

1.2.10 Body text must appear as 9 pt. normal font, and be indented 1 tab from the left and right margins.

1.2.11 “References” must be 9.5 pt. font, bolded, and be aligned with the left margin. Individual entries must be 9 pt. font and should follow American Psychological Association (APA) formatting guidelines. Any lines of text for a single entry that exceed the first line should use a “hanging indent” of 1.5 tabs from the left margin.

1.3 An appropriate page length for publication in the Journal is approximately 15 pages.

2. Citing

Should follow American Psychological Association (APA) formatting guidelines. Click http://jfhb.dusit.ac.th/flie/Ref%20Guidelines. pdf to see the example.

3. Ordering of Titles in Journal of Food Health and Bioenvironmental Science

The written manuscript may contain only English. The content should be easy to understand and clear. If the author uses abbreviation, full word must appear before any abbreviation.

3.1 The title should be brief, the length should not exceed 100 characters.

3.2 The authors if there are more than six authors only the first author is listed, followed by "et al."

3.3 Affiliated entities associated with the author should appear in English languages.

3.4 The abstract must be written in English language. The abstract should briefly summarize the research and not exceed 250 words or 15 lines of text.

3.5 The "Keywords" section must contain no more than four keywords that allow for appropriate searching and selection based upon the article's topic.

3.6 The "Introduction" section should provide background information relevant to the research, provide information regarding the manuscript's content and state the objectives of the work.

3.7 The "Materials and methods" section delineates the procedures, how the research was conducted, sampling method (i.e. simple random samples) and population, and the creation and development of research tools used for data collection and analysis.

3.8 The "Results" section or "Results and Discussion" presents data obtained during the research and may be displayed as tables, graphs, illustrations, and accompanying explanations. Tables should be not have left and right borders and are normally black and white printed. No more than five tables should be present in the "Results" section. Pictures within the section should be clear and use simple black and white coloring with an accompanying caption, the author wishes to use colors for any item they may do so; however, the author will be responsible for the additional costs of color printing.

3.9 The "Discussion" section or "Result and Discussion" should explore the significance of the results of the work and address whether or not the data support the research hypothesis and compare research findings to other similar research works.

3.10 The "Conclusions" section should summary of the main topic covered or a re – statement of the research problem.

3.11 The "Acknowledgements" (if any) section should provide help during the research (e.g., providing materials, laboratory, equipment, etc.) and funding.

Sending Original manuscript

1. Compose the manuscript using the format of the Journal of Food Health and Bioenvironmental Science.

2. Send the manuscript via ScholarOne website https://mc03.manuscriptcentral.com/jfhb

Journal of Food Health and Bioenvironmental Science Publication Ethics

Editorial Regulations

- It is the duty of the Editors to consider the submitted manuscripts related to field of food, health, biological and environmental disciplines and other related fields. The consideration will be based solely on the content. The ethnicity, country of origin, gender, sexual orientation, political affiliation, or religious belief of authors does not influence the editor's decision.
- Throughout the submission, the editors must not share the information about the submissions to anyone except the authors, reviewers and JFHB staffs.
- Editors must make sure the manuscript has no substantial vested interests authors or affiliated organizations.
- The editorial staff have to assure that the manuscript has been peer-reviewed by at least two reviewers in the field of Food, Health, biological and environmental disciplines or other related field appropriate for each manuscript. The editorial staffs also have to be careful about the copyright Infringement, falsification of data, and plagiarisms. If there is an offense according to the said regulations, the editor must investigate and seek for evidence before consider reject the manuscript.
- In case that the Editors suspect that the manuscript might have been published elsewhere, the editor should investigate and consider reject.
- In case of unethical publishing practices that are later uncovered, the action will still be taken seriously.

Reviewer Regulations

- Reviewers are expect to give constructive and professional comments. Improper criticism must be avoided.
- If the manuscript given is lies beyond area of expertise, the reviewers should inform the staff immediately.
- Reviewers must keep the manuscript confidential. Do not share any information of the manuscript to anyone other than the editorial staff.
- In case that the reviewers find that the other works contained in the manuscript are not well credited, reviewers are required to inform the editorial staff.
- If there are conflicts of interests, reviewers should inform the editorial staff. Editors will decide whether the reviewer is appropriate for the manuscript or not.

Author Regulations

- The authors should write the manuscript related to the theme of Food, Health, biological and environmental disciplines. The research manuscript should contained relevant background information, efficient methodology, APA style citation, accurate results, and reasonable discussion.
- The authors should follow the journal guidelines strictly.
- Any opinion or perspective made in the manuscript must be explicitly highlighted as "opinion" or "perspective"
- The authors must be careful and aware that fraudulent information and omission of important information are unethical author behaviors.
- The authors must be able to provide research data if the Editor see needed.
- Authors must reference other works properly. Any work involved in the manuscript also must be well credited.
- The authors must make sure that the manuscript has not been published elsewhere before and is not currently in the publication process in other journals.

- The person must have made significant contributions to the manuscript, participate and give important efficient content during revisions and provide approval for publication in order to be listed as an author. Researchers who do not meet the above criteria should be listed in the Acknowledgements section.

- Author should identify any conflicts of interest that might have influenced the data and/or interpretations of data.

- To make the efficient revision, the authors should respond to all the given critiques and suggestions during the revision.

- If the authors find errors in their works that need to be correct, the author should inform the editors immediately.