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Content

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Liposomes Encapsulating *Artocarpus lakoocha* Roxb. and *Glycyrrhiza glabra* L. Extracts: Characterization and Shelf Life of Freeze-Dried Vesicles

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Abstract

Liposome is the one way of encapsulation of extracts for reducing the extract degradation. This study was to prepare the liposome entrapped extracts of *Artocarpus lakoocha* Roxb. (L-Al), *Glycyrrhiza glabra* L. (L-Gg) alone and in combination of *A. lakoocha* and *G. glabra* extracts (L-AlGg). The liposomes were prepared by Mechanochemical method and freeze-drying. For stability of liposomes, storage at 4, 25 and 45°C for 8 weeks was performed. The trapping efficiency of liposomes and tyrosinase inhibitory activities of extracts entrapped in liposome were investigated. Results showed liposome morphology was the spherical vesicles evaluated by TEM. Before freeze-drying, liposomes had particle sizes of 156.966 ± 0.808, 140.8 ± 0.818 and 158.633 ± 4.193 nm for L-Al, L-Gg and L-AlGg, respectively. The entrapment efficiency of L-Al, L-Gg and L-AlGg was found to be 95.83 ± 13.48, 97.99 ± 5.23 and 93.90 ± 16.28 %, respectively. The tyrosinase inhibitory activities of released extracts from L-Al, L-Gg and L-AlGg were 81.57 ± 1.22, 68.92 ± 1.23 and 81.40 ± 0.64 %, respectively. After freeze-drying, the particle sizes of L-Al and L-AlGg were no significant changes, while L-Gg particle size was bigger ($p < 0.01$). The liposome entrapment and tyrosinase inhibitory activity of released extracts were not significantly changed after freeze-drying. This indicates good stability and no extract leakage of liposomes. In storage at 4°C for 8 weeks, the entrapment efficiency of L-Al, L-Gg, L-AlGg and tyrosinase inhibitory activity of released extracts were not significantly different, comparing with controls. When increasing temperature of storage effected on the significantly reduction of the entrapment of liposomes and the tyrosinase inhibitory activity of released extracts ($p < 0.01$). Therefore, the freeze-dried liposome and storage at low temperature is recommended for stabilizing liposome and extract quality.

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Introduction

Liposomes are small artificial vesicles of spherical shape that can be formed by the self-assembly of phospholipids in aqueous solution. They have been useful as a drug or food carrier because they are able to retain water-soluble substances in the inner aqueous phase and oil-substances in the bilayer wall (Bangham & Horne, 1964; Bangham et al., 1965; Takahashi et al., 2007). Liposomes also have proved to be applied carriers for gene or compound delivery to cells in culture and in preclinical trials. They are extensively used as carriers for numerous molecules in cosmetic industries and have been studied the use of liposome encapsulation to grow delivery systems that can entrap unstable compounds such as antioxidants, antimicrobials and other bioactive compounds (Inoh et al., 2004; Atrooz, 2011; Benech et al., 2002). Liposomes can trap both hydrophobic and hydrophilic compounds, avoid decomposition of the entrapped combinations, and release the entrapped into deep skin (Shehata et al., 2008). This system has been reported to effectively enhance the skin permeation of plant extracts (Tasahashi et al., 2009; Pinsuwan et al., 2010). For freeze-dried liposomes, lyophilization still remains the main studied technique even if examples of marketed lyophilized drug or cosmetic products are very limited. This is due to the complexity of the process since the choice of excipients and process parameters to protect the membrane integrity from stresses due to freezing and dehydration is challenging (Jensen, 2017; Franzé et al., 2018). The cryoprotectants (lactose, trehalose and sucrose) are considered excipients of choice to include in outer aqueous phase of liposomal dispersions for stabilizing the liposome membrane during freeze-drying and reducing the detrimental effects (Viswanathan et al., 2019; Kannan et al., 2015; Wang et al., 2006).

A. lakoocha is a tropical tree widely distributed throughout the regions of Southeast such as Sri Lanka, India, Myanmar, Vietnam, Indonesia, Malaysia and Thailand. *A. lakoocha* has been used for parasite treatment and the ethanolic extract of *A. lakoocha* heartwood was reported to possess antioxidant and tyrosinase inhibitory activities (Sritularak et al., 1998; Singhatong et al., 2010; Panichakul et al., 2018). The main active compounds in *A. lakoocha* heartwood extract are oxyresveratrol and resveratrol (Likhitwitayawuid et al., 2006; Tengamnuany et al., 2006; Panichakul et al., 2018). Encapsulation of *A. lakoocha* extract into liposomes was able to provide prolonged release. A vivo skin whitening study demonstated that the lotion containing *A. lakoocha* extract-loaded liposomes exhibited better skin whitening effects on human volunteers than the lotion containing non-encapsulated *A. lakoocha* extract (Teeranachaideekul et al., 2013).

Licorice, the root of the *Glycyrrhiza glabra* as herbal medicine in Asia and southern Europe is known to have anti-oxidant, anti-melanogenic, anti-inflammatory, anti-viral, anti-allergic and anti-cancer activities (Mukhopadhyay & Panja, 2008; Ha et al., 2013; Han et al., 2013; Bae et al., 2014). Glabridin, the main component of licorice has tyrosinase inhibitory activity to skin-whitening in cosmetic products, and is used in dietary supplements, foods (Simmler et al., 2013). The ethanolic extract of *G. glabra* roots was incorporated in liposomes and hyalurosomes to promote the proliferation and migration of fibroblasts, favouring the closure of the scratched area, and reduced inflammation, favouring the re-epitelization process (Castangia et al., 2015). In addition, Liposomes of *G. glabra* extract had been developed for enhancing the anti-oxidative protection, the immune-modulating activity and anti-tubercular drugs of *G. glabra* (Castangia et al., 2015; Zhang et al., 2013; Viswanathan et al., 2019; Wu et al., 2017).

This study involves the development of liposome dry powder containing extracts of *A. lakoocha, G. glabra* or their mixtures for the cosmetic application. The physicochemical properties of liposomes, such as morphology, size and stability on storage were evaluated.

Materials and methods

1. Plant materials and extraction

A. lakoocha heartwoods (Al) and *G.glabra* roots (Gg), plant powders were purchased from Vejpongosot drugstore in Bangkok, Thailand. The plants powders were extracted in ethanol by maceration modified from previously described (Panichakul et al., 2018). Briefly, 200 g of plant powder was macerated in 1 liter of 95% ethanol at room temperature for 6 h. After three times of maceration, the alcoholic extracts were pooled, filtrated and evaporated under reduced pressure below 45°C. *A. lakoocha* and *G.glabra* extracts were kept at -20°C until used.

2. Determination of total phenolic content

The total phenolic compound in Al and Gg extracts was determined by Folin-Ciocalteu reagent, (Javanmardi et al., 2003). In 96-well plates, 4.5 µl of 1 mg/mL of

samples diluted with 126 µl of deionized water (DI) was mixed with 90 µl of 2% Na_2CO_3 for 3 min and then added with 4.5 µl of 50% Folin-Ciocalteu reagent. After the samples were incubated at room temperature for 30 min, resulting in a blue molybdenum-tungsten complex that was determined at 750 nm by microplate reader (Biochrom EZ Read 2000). The total phenolic content in each sample was calculated by comparing to standard gallic acid and shown as milligrams of gallic acid equivalents to one gram of extract (mg GAE/g extract).

3. Mushroom tyrosinase assay

The inhibition of mushroom tyrosinase activity was determined using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate (Ko et al., 2013). Briefly, 20 µl of samples and 140 µl of 20 mM phosphate buffer (pH 6.8), and 20 µl of 461.68 unit/ml of mushroom tyrosinase were added to each well of a 96-well plate and mixed with gentle shaking at room temperature for 10 min. Then, 20 µl of 4 mM L-DOPA was added and incubated at 37 °C for 30 min. The relative amount of dopachrome formed in the mixture was determined at a wavelength of 475 nm by microplate reader (Biochrom EZ Read 2000). Tyrosinase and L-DOPA solution was used as the control and kojic acid at various concentrations of 0.0024 – 1.25 mg/ml was a positive control. The inhibition of tyrosinase activity was calculated and expressed as a percentage of control.

4. Liposome preparation

Liposome preparation was performed by the mechanochemical method using high speed homogenizer modified from a previous report (Takahashi et al., 2007). Soybean lecithin 20 g was added into deionized water 150 ml, and then mixed for 5 min into ultrasonic bath (Transsonic digitals, Elma, USA). This mixture was dispersed by high speed homogenizer (Ultra Turrax T25, IKA Labortechnik, USA) for 10 min at 11,000 rpm. After adding with 10 ml of propylene glycol, 40 ml of 1 g/ml trehalose in water and 1 ml Verstatill PC, the mixture was then continuously homogenized for 15 min at 22,000 rpm. Liposomes were determined particle size by Zetasizer Nanoseries model S4700 (Malvern Instrument, UK).

For preparation of liposomes encapsulating extracts of *A. lakoocha* (L-Al), *G. glabra* (L-Gg) or a combination of Al and Gg extracts (L-AlGg), 0.2 g of Al and Gg alone or combined extracts (Al : Gg at a ratio 9:1) were separately suspended into 10 ml propylene glycol and 70 ml deionized water. Eighty milliliters of extracts and 80 ml of soybean lecithin were mixed for 5 min into ultrasonic bath. The mixtures were dispersed by high speed homogenizer for 10 min at 11,000 rpm, then added with 40 ml of 1 g/ml trehalose and 1 ml Verstatill PC, and continuously homogenized for 15 min at 22,000 rpm, respectively.

5. Measurement of particle size and morphology of liposomes

The Z-average particle size (hydrodynamic diameter), size distribution and polydispersity index (PI) of liposomes were determined by Dynamic laser light scattering method using Zetasizer Nanoseries model S4700 (Malvern Instrument, UK) with a wavelength of 532 nm at 25°C. The scattering angle was fixed at 90°. Prior to analysis, 0.1 ml of fresh or rehydrated liposomes suspension sample was diluted with 10 ml of deionized water. Particle size of liposomes were also monitored during leakage and experiments carried out at 4, 25 and 45°C for 8 weeks to assess the stability of liposome formations in storage conditions. Each measurement was repeated three times.

The morphology of liposomes was detected by Transmission electron microscopy (TEM). Liposome suspension was diluted at 1:10 with deionized water. One drop of diluted sample was left alone on a copper grid for 5 min and stained with 1% phosphotungstic acid for 2 min. After the excess, liquid was absorbed by the filter papers, the stained sample was air-dried at room temperature and then observed under TEM (JEOL, JEM-1400, Japan).

6. Determination of the entrapment efficiency of liposomes

To assess the entrapment efficiency (EE) of L-Al, L-Gg and L-AlGg, a phenolic compound used as a marker was determined by Folin-Ciocalteu assay. Briefly, 1 ml of each liposome suspension was centrifuged at 13,000 rpm for 15 min. The floated liposomes were collected and added 1 ml of ethanol and 1 ml of hexane. After shaking for 15 min, samples were centrifuged at 5,000 rpm for 15 min. The ethanol parts were collected and then determined the total phenolic contents. In 96-well plates, 4.5 µl of samples diluted with 126 µl of deionized water (DI) was mixed with 90 µl of 2 % Na_2CO_3 for 3 min and then added with 4.5 µl of 50 % Folin-Ciocalteu reagent. After 30 min of incubation, a blue molybdenum-tungsten complex was determined at 750 nm by microplate reader (Biochrom EZ Read 2000, UK). The total phenolic content in each sample was calculated by comparing to standard gallic acid. The EE% was calculated from the total phenolic contents of

incorporated extract divided by the total phenolic contents of extracts used at the beginning of preparation multiplied by 100.

7. Lyoprotection of liposomes

Freshly prepared liposomes with or without the adding of extracts were mixed with trehalose. Liposomes suspension was quickly frozen with iced acetone, stored in freezer at -20°C 48 h. After that frozen liposomes were dried for 48 h using Supermodulyo-230 freeze dryer (Thermo, USA) with a condenser temperature of -55°C and pressure at $1\text{-}10^{-1}$ mbar. The dried samples were stored at 4°C.

8. Stability of liposome formation

After lyoprotection of liposomes, the stability of dried liposomes was studied. The samples of dried liposomes kept for 8 weeks at 4, 25 and 45°C were analyzed and compared to freshly prepared liposomes as controls.

Two grams of each dried L-Al, L-Gg and L-AlGg were in glass bottles sealed with foil and then kept at 4, 25 and 45°C for 8 weeks. To determination of particle size of liposome after 8 weeks of storage, 0.1 g of lyophilized liposomes were reconstituted in 400 µl of 2% xanthan gum solution and diluted at 1:100 with deionize water. The liposome suspension was determined particle size by Dynamic laser light scattering method.

After storage for 1, 2, 4, 6 and 8 weeks, one hundred milligrams of dried liposomes were reconstituted in 400 µl of deionize water, added 400 µl of ethanol and 400 µl of hexane. After shaking for 15 min, the mixtures were centrifuged at 5,000 rpm for 5 min. The ethanol part was collected and used to determine total phenolic contents by Folin-Ciocalteu assay and tyrosinase inhibitory activity by mushroom tyrosinase assay. The color of freeze-dried liposomes at 8 weeks was determined by Chroma meter (Konica Minolta, CR-400).

9. Statistical analysis

The data were analyzed by the SPSS version 17. These experiments were expressed as mean values ± standard deviation (SD). Data were subjected to statistical analysis using Paired – Samples T Test and One-way ANOVA, and p values < 0.01 were regarded as significant.

Results and discussion

The total phenolic contents and tyrosinase inhibitory activity of extracts from *A. lakoocha* heartwood (Al) and *G. glabra* root (Gg) were determined. Results showed that the total phenolic contents of Al and Gg alone and in combination with Al and Gg at a ratio of 9:1 were 103.42 ± 0.71, 75.03 ± 0.57 and 97.80 ± 5.16 mg GAE/g extract, respectively. The inhibitory effects of Al and Gg alone and in combined Al and Gg extracts on mushroom tyrosinase were analyzed and presented as the concentration that inhibited 50% of the mushroom tyrosinase activity (IC_{50}) (Table1). Al and Gg alone and in combined Al and Gg extracts inhibited mushroom tyrosinase activity with IC_{50} values of 0.074 ± 0.040, 0.137 ± 0.065 and 0.071 ± 0.028 mg/ml, respectively. These values indicate that the Al, Gg alone and in combined Al and Gg extracts were more potent than kojic acid (IC_{50} = 0.256 ± 0.005 mg/ml) as tyrosinase inhibitors. Al alone and combined Al and Gg extracts had higher tyrosinase inhibitory activities than those of Gg extracts in Table 1. The bioactivity of Al and Gg extracts as known is tyrosinase inhibitory activity (Povichit et al., 2010; Velvizhi & Annapurani, 2018; Panichakul et al., 2018). Our previous reports found that the tyrosinase inhibitory activity of extracts from Al alone and combined Al and Gg extracts was higher than those of Gg extracts (Panichakul et al., 2018).

Table 1 The total phenolic contents and tyrosinase inhibitory activity of extracts from *A. lakoocha* heartwood (Al) and *G. glabra* root (Gg) and in combination of Al and Gg

Extracts	Total phenolic contents mg GAE/g extract	Tyrosinase inhibitory activity (IC_{50}) (mg/ml)
Al	103.42 ± 0.71	0.074 ± 0.040*
Gg	75.03 ± 0.57	0.137 ± 0.065*
Al and Gg	97.80 ± 5.16	0.071 ± 0.028*
Kojic acid	-	0.256 ± 0.005

Remark: Data are mean ± S.D. from three independent experiments.
* indicates significant different at $p < 0.01$.

1. Production of liposomes of Al, Gg alone and in combined Al and Gg extracts

Al and Gg alone and in combination of Al and Gg extracts were encapsulated in liposomes by the mechanochemical method using high speed homogenizer. Liposomes containing extracts were evaluated for particle size and morphology, % entrapment and stability of liposome formation. TEM images showed liposomes were spherical in shape (Fig. 1) as similar as previously described (Rangsimawong & Ngawhirunpat, 2015). The particle sizes of empty liposomes and liposomes entrapped Gg extract (L-Gg) were 138.133 ± 0.35 and 140.8 ± 0.818 nm, respectively that was

slightly smaller than particle sizes of liposomes entrapped with Al alone (L-Al) and in combination of Al and Gg extracts (L-AlGg) (156.96 ± 0.80, and 158.633 ± 4.193 nm, respectively) (Table 2). The entrapping efficiency of the individual liposome, L-Al, L-Gg and L-AlGg was 95.83 ± 13.48, 97.99 ± 5.23 and 93.90 ± 16.28 %, respectively (Table 3). Results indicated that no significantly different efficiency of liposome entrapment of Al and Gg alone and in combination of Al and Gg extracts. According to liposomes composed of phospholipid bilayers and an aqueous cavity has been reported to effectively enhance the skin permeation of plant extracts (Takahashi et al., 2009). Encapsulation of Al and Gg extracts in liposomes was able to enhance the quality of extracts bioactivity as previously described (Teeranachaideekul et al., 2013; Wu et al., 2017).

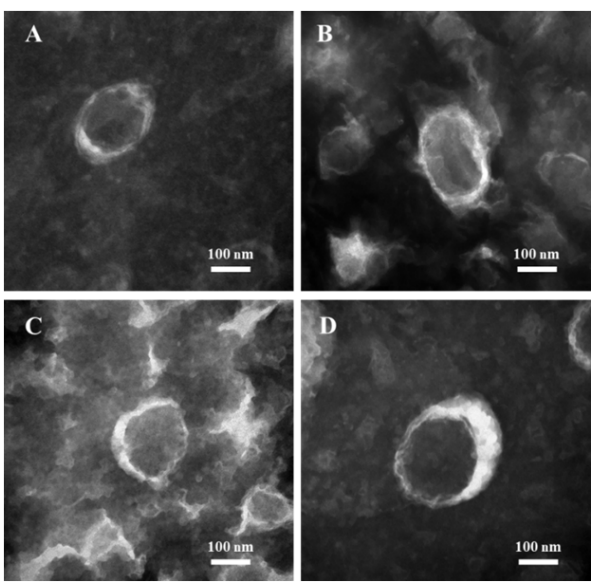

Fig. 1 TEM (Transmission electron microscopy) images of liposomes A) empty liposome; B), C) and D) liposomes entrapped Al, Gg alone and in combined Al and Gg extracts, respectively. Magnification x 300,000

Table 2 Mean particle size and polydispersity index of liposomes before and after freeze-drying

Liposomes	Before Freeze-drying		After Freeze-drying	
	Particle size (nm)	Polydispersity Index : PDI	Particle size (nm)	Polydispersity Index : PDI
L-Empty	138.133 ± 0.350	0.157 ± 0.004	ND	ND
L-Al	156.966 ± 0.808	0.201 ± 0.018	163.5 ± 2.95	0.301 ± 0.021
L-Gg	140.8 ± 0.818	0.154 ± 0.019	151.40 ± 0.96*	0.222 ± 0.009
L-AlGg	158.633 ± 4.193	0.204 ± 0.018	180.6 ± 2.26	0.343 ± 0.014

Remark: Data are mean ± S.D. from three independent experiments.
* indicates significant different at $p < 0.01$.
ND is not done.

Table 3 Percentages of liposome entrapment before and after freeze-drying

Liposomes	% of Encapsulation Efficiency	
	Before freeze-drying	After freeze-drying
L-Al	95.833 ± 13.480	83.333 ± 1.990
L-Gg	97.992 ± 5.230	94.779 ± 5.016
L-AlGg	93.898 ± 16.280	90.847 ± 2.560

Remark: Data are mean ± S.D. from three independent experiments.
* indicates significant different at $p < 0.01$.

2. Freeze-drying of liposomes

To overcome the instability of liposomes, the freeze-drying of liposomes with adding cryprotectants (lactose, trehalose and sucrose) was previously reported. Trehalose at a lipid : cryoprotectant ratio of 1:4 was found to be the most suitable as compared to lactose and sucrose (Viswanathan et al., 2019). In this study, trehalose was used as a cryprotectant to achieve reproducible results after freeze drying liposomes. All freeze dried liposomes, L-Al, L-Gg and L-AlGg (Fig. 2) were redispered on reconstitution and then determined particle size, % entrapment and tyrosinase inhibitory activity as shown in Table 2, 3 and 4. Results showed after freeze drying, there was no significant change in the particle size of L-Al and L-AlGg, but particle size of L-Gg was bigger ($P < 0.01$) (Table 2). In addition, % entrapment and tyrosinase inhibitory activity of released extracts were not significantly changed after freeze-drying reconstitution (Table 3 and 4). These results indicate that freeze-dried liposomes were stable and no significantly extract leakage from liposome particles was found. Thus, the cryprotectants on the nature of freeze-dried liposomes was able to achieve reproducible results after freeze drying of liposomes and reduce the aggregation of liposomes as previously reported (Viswanathan et al., 2019; Mohammed et al., 2006; Yang et al., 2013).

Fig. 2 Before and after freeze-drying of liposomes

Table 4 Tyrosinase inhibitory activity of extracts released from liposomes before and after freeze drying

Liposomes	%Tyrosinase inhibition/ 1 mg/ml extract	
	Before freeze-drying	After freeze-drying
L-Al	81.570 ± 1.220	77.223 ± 1.841
L-Gg	70.340 ± 1.229	68.070 ± 2.500
L-AlGg	81.400 ± 0.640	79.920 ± 1.700

Remark: Data are mean ± S.D. from three independent experiments.
* indicates significant different at $p < 0.01$.

3. Long-term stability of freeze-dried liposomes of L-Al, L-Gg and L-AlGg

Freeze-dried liposomes of L-Al, L-Gg and L-AlGg were stored for 8 weeks at different temperatures of 4, 25 and 45°C for evaluating the liposome stability. The particle size, % entrapment and tyrosinase inhibitory activity were determined in week 1, 2, 4, 6 and 8 as shown in Table 5 and Fig. 3 and 4. Results showed after storage for 8 weeks, at 4, 25 and 45°C, particle sizes of L-Al and L-Gg were significantly changed ($P < 0.01$), compared with those before storage as controls. For L-AlGg, its particle size had no change when was kept at 4°C, but storage at 25, 45°C, the particle size was significantly smaller ($P< 0.01$), compared with a control (Table 5). At 8 weeks of storage, the color of freeze-dried L-Al, L-Gg and L-AlGg kept at 4°C was not changed, while these liposomes kept at 25 and 45°C had more color intensity, compared with controls. The entrapment efficiency of L-Al, L-Gg and L-AlGg evaluated during 8 weeks (at 1, 2, 4, 6, and 8 week) was found that all liposomes kept at 4°C had no significantly difference ($P < 0.01$), compared with those before storage as controls. At 25 and 45°C, the % entrapment of all liposomes was reduced as shown in Fig. 3. In addition, tyrosinase inhibitory activity of released Gg and in combined Al and Gg extracts had no change when kept at 4°C compared with controls, while the released-Al extract had significantly reducing tyrosinase inhibitory activity ($P < 0.01$). When temperature of storage was up to 25 and 45°C, the tyrosinase inhibitory activity of all released extracts was significantly reduced, compared with those activities of extracts as controls ($P < 0.01$) (Fig. 4). This indicates that the increasing temperature for liposome storage was able to effect on instability of liposomes while the low temperature, at 4°C is still to keep the quality of liposomes. As a previous report, auto-oxidation of beta-carotene in liposomes kept at low temperature (4°C) was reduced beta-carotrene (Moraes et al., 2013). Interestingly, the combination of Al and Gg extracts could be encapsulated in the same vesicle of liposomes. The bioactivity of released L-AlGg extracts in tyrosinase inhibition was the same level before storage, while tyrosinase inhibitory activity of released- L-Al extracts after storage was reduced. Therefore, the encapsulation of combined *A. lakoocha* and *G. glabra* extracts in liposome is a considered technique for liposome development. In further study, liposomes of Al, Gg alone or in combination of Al and Gg extracts will be applied to develop cosmetic products and evaluation of the efficiency in human volunteer. Finally, this finding is an alternative way for improving quality and enhancing the shelf-life of liposomes.

Table 5 Mean particle size and polydispersity index of liposomes before and after storage at 4, 25 and 45°C for 8 weeks

Liposomes	Temperature (°C)	Before storage		After storage for 8 weeks	
		Particle size (nm)	PDI	Particle size (nm)	PDI
L-Al	4	163.5 ± 2.959	0.30 ± 0.021	184.7 ± 4.222*	0.33 ± 0.005
	25			201.3 ± 1.4*	0.39 ± 0.007
	45			341.633 ± 3.394*	0.370 ± 0.070
L-Gg	4	151.4 ± 0.960	0.222 ± 0.010	168.267 ± 1.193*	0.288 ± 0.026
	25			170.067 ± 0.709*	0.39 ± 0.018
	45			135.933 ± 2.138*	0.255 ± 0.019
L-AlGg	4	180.6 ± 2.260	0.34 ± 0.014	186.367 ± 3.360	0.33 ± 0.025
	25			163.267 ± 2.272*	0.32 ± 0.022
	45			149.367 ± 1.157*	0.304 ± 0.032

Remark: Data are mean ± S.D. from three independent experiments.
* indicates significant different at $p < 0.01$.

Conclusion

The encapsulation of Al and Gg alone or in combination of Al and Gg extracts in liposomes is an alternative way to enhancing the delivery systems of extracts. The long-term stability of liposomes can be improved by formulating the systems as freeze-dried products. To reduce liposome aggregation, carbohydrates such as trehalose is required in systems of freeze-drying. In addition, the low temperature of liposome storage is able to keep liposome quality.

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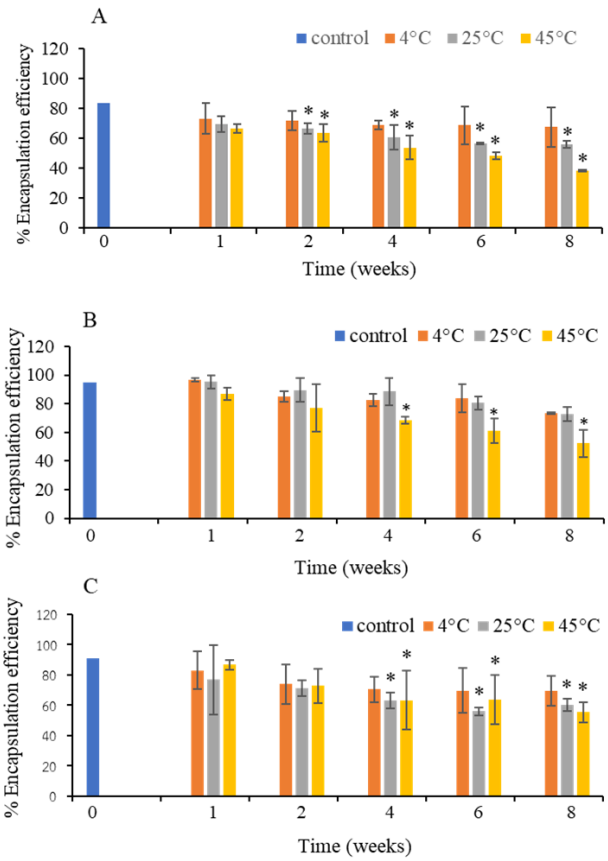

Fig. 3 Encapsulation efficiency of L-Al, L-Gg and L-AlGg after kept for 8 weeks at 4, 25 and 45°C. A) L-AL, B) L-Gg and C) L-AlGg, liposomes were determined the % entrapment in 1, 2, 4, 6 and 8 weeks. Data are mean ± S.D. from three independent experiments. * indicates significant different at $p < 0.01$

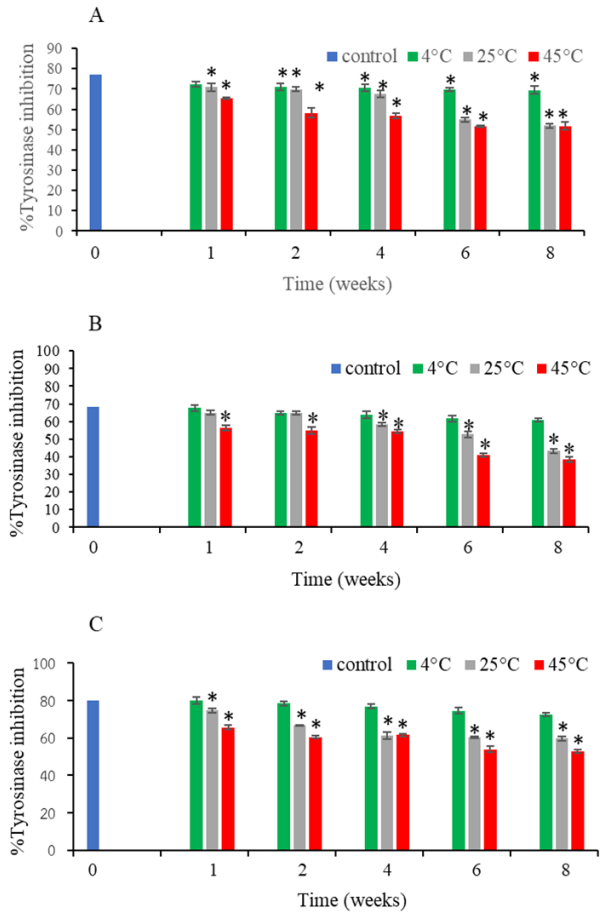

Fig. 4 Tyrosinase inhibitory activity of released extracts from L-Al, L-Gg and L-AlGg after kept for 8 weeks at 4, 25 and 45°C. A) L-AL, B) L-Gg and C) L-AlGg, liposomes were evaluated the % of tyrosinase inhibitory activity in 1, 2, 4, 6 and 8 weeks. Data are mean ± S.D. from three independent experiments. * indicates significant different at $p < 0.01$

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Total Phenolics, Flavonoids, Anthocyanins and Antioxidant Activities of Khaow-Mak Extracts from Various Colored Rice

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Abstract

Khaow-Mak is fermented food of rice, which contains a lot of nutrients and antioxidant bioactive compounds. Generally, Khaow-Mak is fermented from cooked white glutinous rice. However, it can be fermented with colored rice (black, purple and red pericarp colored grains) in order to increase bioactive compounds and antioxidant performance. The study was conducted to investigate the chemical composition of Khaow-Mak extracts, total phenolic, flavonoid and anthocyanin contents. The antioxidant activities were evaluated by using the scavenging towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). The colored rice was collected from 16 local colored rice (red, black and purple) around Thailand. The cooked rice was fermented with a starter (Look Pang) at room temperature for 5 days. Fermented rice samples were extracted with 95% ethanol for 24 hours. Dried crude extracts were obtained using a rotary evaporator at 45°C. The results showed that the content of bioactive compounds of all colored rice were increased after fermenting time. Leum Phua glutinous rice had the highest contents of total phenolic (45.66±0.01 mgGAE/g) flavonoid contents (39.35±0.07 mgRE/g) and anthocyanin contents (3.37 ± 0.21 mg/g). In addition, Leum Phua glutinous rice showed the hightest antioxidant activity of DPPH (EC_{50} 0.364±0.02 mg/ml) and FRAP (28.018±0.01 mgFe(II)/g. These results potentially support the use of local rice extracts as the active raw material of functional food and/or cosmetics.

Introduction

Colored rice (pigmented rice) is a kind of brown rice obtained by removal of husk. Pigmented rice is distinguished by the rice grain having red brown or dark purple color in its covering layers. Pigments, which are located in the aleurone layer of rice grain, have been reported as a mixture of anthocyanin compounds, which belong to the family of flavonoids (Yawadio et al., 2007). Flavonoids, the major class of phenolic compounds in plants, can be divided into different classes, being the anthocyanidins the most common. Generally, the anthocyanidins are bound to glycosides, which are called anthocyanins (Kong et al., 2003). The phenolic compounds have been found as a major active component

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for antioxidation (Iqbal et al., 2005). Several compounds have already been identified in this cereal, mainly phenolic acids and anthocyanins (Oki et al., 2002).The anthocyanin plays an important role in antioxidant (Sutharut & Sudarat, 2012) which is natural phenolic pigments that was reported to scavenge free radicals such as superoxide (O_2^-), singlet oxygen ($^{\cdot}O_2$), peroxide (ROO^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Wang & Jiao, 2000b). Colored rice is reported as a potent source of phenolic compounds (polyphenols) which has greater amount comparing with white rice and it contains a lot of nutritional advantages over white rice (Vichapong et al., 2010). The total polyphenols content in rice exist in the soluble form representing 40% in light brown rice grains and around 81% in red and black pericarp color grains. (Mira et al., 2009). In addition, the pigmented rice has higher DPPH radical-scavenging activity than white rice due to the polymeric procyanidins which are the major component for antioxidant (Oki et al., 2002).

Fermented sweet rice, which is called Khaow-Mak, is known as one of the famous traditional food from Thai folk wisdom in Thailand produced from the process of fermentation using microorganisms. The traditional starter culture (Look Pang) contains yeast, mold and herbs, which is used for fermenting cooked white glutinous rice (Manosroi et al., 2011). Enzymes from the molds hydrolyze starch in the rice which turns into sugars, which are partially fermented into alcohol by the yeast. Organic acids (e.g. lactic acid) are also produced (Lotong, 1992). Black glutinous rice is sometimes substituted for white glutinous rice to produce Khao-Mak since it is a rich source of phytochemicals such as anthocyanins (Sompong et al., 2011). Khaow-Mak has been documented as a rich source of probiotics and bioactive compounds, offering various food properties that influence human health. Several studies showed that the fermentation can increase bioactive compounds, such as polyphenolics, flavonoids, phytic acid, γ-oryzanol and vitamin E. Besides, the degradation of antinutritional compounds to phytic acid was also found in antioxidative activity. The fermentation can lead to the improved nutritional quality of food (Zhai et al., 2015; Plaitho et al., 2013; Michela et al., 2019). Rice (*Oryza sativa* L.) grain has been reported to contain several groups of antioxidants, including phenolic compounds, flavonoid, and anthocyanin (Iqbal et al., 2005). These compounds have been especially rich in pigmented rice (black or red pericarp) (Kehrer, 1993). Antioxidants are defined as organic molecules that promote health by protecting the body's cells from damage caused by free radicals and reactive oxygen species that may otherwise exert harmful metabolic effects. It has been widely accepted that an excess of generation of free radicals leading to oxidative damage, which are responsible for the age-related damage at cellular and tissue levels (Fusco et al., 2007). Thus, a balance between oxidant and antioxidant is necessary in order to reduce the rate of formation of aging changes and disease pathogenesis (Rohrer & Siebenmorgen, 2004).

In this study, Khaow-Mak was produced from 16 varieties of rice. The aims are to evaluated the concentration of bioactive compounds, total phenolics, flavonoids and anthocyanins content. The total antioxidant capacity determined by the DPPH method compared with those determined by FRAP method. In addition, the comparison of bioactive compounds and antioxidant activities from various colored Khaow-Mak extracts between before and after fermentation. The extracts with antioxidant activity are fundamental to the development of health and beauty products further, which can be applied to the active ingredient in cosmetic, food and other industries.

Materials and methods

1. Preparation of crude extracts from Khaow-Mak

The glutinous rice of Leum Phua rice (Tak province), Khao Kam (Chiang Rai province), Khao Kam Doi (Phayao province), Khaoneow Damhmo (Phatthalung province), Khaoneow Dam (Buri Ram province) and Khaoneow Dang (Loei province). The nonglutinous rice of Hommali Dang rice (Saraburi province), Hom Nin rice (Lopburi province), Rice Berry rice (Pathum Thani province), Homnin-Jakkapat rice (Ubon Ratchathani province), Sang Yod rice (Phatthalung province), Mali Nin rice (Surin province), Niang Guang rice (Buri Ram province), Tubtim Chumphae rice (Khon Kaen province), Homdam Sutabut rice (Chiang Rai province) and Hommali Dam rice(Chiang Rai province), all of these are rice varieties used for experiment. The rice was soaked with water for 6 hours. It was mixed with distilled water (1:3 w/v) and cooked with the ordinary rice cooker. Cooked rice was cooled at room temperature and fermented with 0.5% Look-Pang (0.5g/100 g of raw rice) at room temperature for 5 days in a glass container. The fermented rice was dried in the oven at 60°C for 24 hours. Dried rice samples were extracted with 95%

ethanol under stirring in a shaker at 120 rpm for 24 hours. The ethanol extracts were separated in the centrifuge at 6,000 rpm for 10 min and were filtered through a paper filter (Whatman No.1). The remaining wastes were reprocessed by the same methods and the extracts which were combined well. The extracts were transferred to a flat-bottomed flask. The solvents were evaporated by a rotary evaporator at 45°C until dry samples. All crude Khaow-Mak extracts were stored at -10°C in storage vials for determination of bioactive compounds and antioxidant activities (Plaitho, 2016).

2. Total phenolic content

The total phenolic content (TPC) was determined by the Folin–Ciocalteu method with some modification (Iqbal et al., 2005). 20 g of Khaow-Mak extracts was diluted with 99.99% ethanol. Then, 100 µl of diluted extracts in 8.4 ml distilled water was mixed with 500 µl of freshly prepared diluted Folin–Ciocalteu reagent (0.2 N). After 1 min, 1 ml of 20% sodium carbonate was added. Mixtures were incubated at room temperature for 2 hours in the dark. The absorbance at 760 nm was measured by spectrophotometer. The total phenolic contents were calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per gram of the sample (mg GAE/g dried extract).

3. Determination of Total Flavonoid Content

The total flavonoid content (TFC) was assayed as described by Shen et al. (2009) with minor modifications using rutin as a standard. 10 mg of Khaow-Mak extracts was diluted with 80% ethanol. Then, 1 ml of the extracted samples were put in a 10 ml volumetric flask containing 4 ml of distilled water and mixed with 0.3 ml 5% $NaNO_2$ solutions. After 6 min, 0.3 ml 10% $AlCl_3.6H_2O$ solution was added. Al $(NO_3)_3$ was added to the flask for another 6 min reaction. After another 6 min, 2 ml 1 M NaOH was added. The reaction solution was well mixed, kept for 15 min and the absorbance was determined at 510 nm. Qualification was done using the Rutin as standard and the results was expressed as milligrams of rutin equivalent (mg RE) per gram of the sample (mg RE/g dried extract).

4. Determination of Total Anthocyanin Content

The total anthocyanin content (TAC) was determined by the pH-differential method which bases on the structural changes in chemical forms of anthocyanin and absorbance measurements at pH 1.0 and 4.5. (Giusti & Wrolstad, 2001). 10 mg of Khaow-Mak extracts was diluted with 80% ethanol. Then, 1 ml of Khaow-Mak extracts solution into 10 ml volumetric flask for preparing two dilutions of the sample, one adjust volume with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5, diluting each. Let these dilutions equilibrate for 15 min. Measure the absorbance of each dilution at the 510 and 700 nm (to correct for haze), against a blank cell filled with distilled water. All measurements should be made between 15 min and 1 hr after sample preparation, since longer standing times tend to increase observed readings. Absorbance readings are made against water blanks. The samples to be measured should be clear and contain no haze or sediments; however, some colloidal materials may be suspended in the sample, causing scattering of light and a cloudy appearance (haze). This scattering of light needs to be corrected for by reading at a wavelength where no absorbance of the sample occurs, i.e., 700 nm. Calculate the absorbance of the diluted sample (A) (Sutharut & Sudarat, 2012) as follows:

$$A = (A510 – A700)\ pH\ 1.0 – (A510 – A700)\ pH\ 4.5$$

Calculate the monomeric anthocyanin pigment concentration in the original sample using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

and it was converted to mg of total anthocyanin content /100 g sample. Where MW is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity, calculate pigment content as cyanidin-3-glucoside, where MW = 449.2 and ε= 26,900

5. DPPH radical scavenging activity

The DPPH free radical scavenging activity was carried out according to Fukumoto & Mazza (2000) with some modifications. 0.02 g of Khaow-Mak extracts was diluted with 40 ml of 99.99% ethanol. A series of concentrations of the extract sample at 31.25, 62.50, 125, 250 and 500 mg/ml was prepared. Briefly, 1 ml of each extract was allowed to react with 2 ml of 0.1 mmol/l DPPH solution for 30 min in the dark before the absorbance was read at 517 nm. The radical scavenging activity was calculated as

$$\%\ \text{Inhibition} = [(AB - AA)/AB] \times 100$$

where AA was the absorption of tested extract solution and AB was the absorption of blank sample.

The sample concentration providing 50% effective concentration (EC_{50}) was calculated from the graph ploting inhibition percentage againt sample concentration.

6. Determination of ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay based on the reduction of the Fe(III)-TPTZ complex to the ferrous form was performed according to methods described by Griffin & Bhagooli (2004). Briefly, freshly prepared FRAP reagent was prepared by mixing 0.3 M acetate buffer (pH 3.6) and 10 mM TPTZ solution prepared in 40 mM HCl and 20 mM ferric chloride ($FeCl_3$) at a ratio of 10:1:1 (v/v/v). The 200 µl of Khaow-Mak extracts was mixed with 1.3 ml of the FRAP reagent and after 30 min of incubation at 37°C, absorption was measured at 600 using a spectrophotometer. Aqueous or methanolic solutions of known Fe(II) concentration are used for calibration of the FRAP assay. FRAP values, expressed as mg Fe(II) equivalent/g dried extract (mg Fe(II)/g dried extract).

7. Statistical analysis

All treatments and determinations were implemented in triplicate and the data are expressed as the mean ± standard deviation. One-way analysis of variance followed by Duncan's multiple range tests and T-test were employed for analyzing the variance ($p < 0.05$) of the data.

Results and discussion

Total phenolic content (TPC) of Khaow-Mak produced from colored rice is shown Table 1. The results were found that TPC of Khaow-Mak from colored rice at after fermentation was found higher than before fermentation. Moreover, TPC from black rice and purple rice was found higher than red rice. The TPC of crude Khaow-Mak rice extracts was not clearly differentiated among the black and purple rice varieties with a range of 21.94-45.66 and 21.36-36.02 mg GAE/g, respectively; whereas the red rice varieties showed the lowest of TPC content (19.30-21.03 mg GAE/g). Khaow- Mak produced from Leum Phua glutinous rice had the highest TPC 45.66±0.01 mg GAE /g.The type and concentration of polyphenols in the rice grain vary among genotypes and are related mainly to the pericarp color. Normally, grains with purple and black pericarp colors have a higher concentration of phenolic compounds compared to red pericarp color (Tian et al., 2004; Zhou et al., 2004). Sadabpod et al. (2010) also reported that total phenolic contents of fermented Hom Nil rice and black glutinous rice were higher than those of both raw rice and cooked rice. Similar to other cereal grains, the phenolic compounds in rice exist in the soluble and insoluble (bound) form. However, the grains with red and black pericarp colors were observed higher concentrations of total soluble phenolic compounds (Melissa et al., 2013).

The total flavonoid content (TFC) of the rice samples followed a similar trend to that of TPC. The TFC of fermented rice was higher than that of its corresponding unfermented one (Table 1). Moreover, TFC from black rice and purple rice was found higher than red rice. The highest total flavonoid content (39.35±0.07 mgRE/g) belonged to Leum Phua glutinous rice fermented and the high levels of TFC was also found in other black rice samples. The TFC of crude Khaow-Mak rice extracts from the black, purple and red rice varieties with a range of 13.38-39.35, 16.47-28.03 and 15.30-20.37 mgRE/g respectively. Anthocyanin is well known as the predominant flavonoid in pigmented rice (Kim et al., 2010).

Total anthocyanin content (TAC) of Khaow Mak produced from colored rice is shown Table 1. After the fermentation, the total anthocyanin content of Khaow-Mak were high at day 5 fermentation. Every treatment of rice varieties exhibited a similar trend. Moreover, the total anthocyanin content from black rice and purple rice was found higher than red rice with a range of 1.82-3.37, 2.38-3.27 and 1.32-1.89 mg/g respectively. The TAC of colored rice crude extracts was prominent in the black rice varieties, followed by the red rice varieties. Khaow-Mak produced from Leum Phua glutinous rice had the highest anthocyanin contents 3.37 ± 0.21 mg/g. Mongkontanawat & Lertnimitmongkol (2015) reported that total anthocyanin content of Khaow-Mak fell at day 3 fermentation because acid or weak acid cause partial or total hydrolyzed anthocyanin molecule, finally it dramatically increased again in the end of fermentation. The color of fermented rice (red, black) could be obtained from anthocyanin. Generally, the most widespread anthocyanin from fruit, vegetable and plants is cyaniding-3-glucoside. Abdel-Aal et al. (2006) reported that cyanidin-3-glucoside and peonidin-3-glucoside were identified as two major anthocyanins in pigmented rice, especially black rice.

The DPPH radical-scavenging ability is frequently used to evaluate the hydrogen donating of the antioxidants and the results are expressed as EC_{50} values, indicating

Table 1 Total phenolic, Flavonoid contents and Antrocyanin contents of Khaow-Mak extracts from colored rice

Sample (Color rice varieties)	Extraction Yield (%)	Phenolic (mg GAE /g)		Flavonoid (mgRE/g)		Anthocyanin mg/g	
		Before fermentation	After fermentaion	Before fermentaion	After fermentaion	Before fermentaion	After fermentaion
Glutinous rice							
Leum Phua rice^{B}	17.41^{i}	37.45±0.01^{a}	45.66±0.01^{a*}	35.64±0.01^{f}	39.35±0.07^{a*}	2.48±0.35^{f}	3.37 ± 0.21^{a*}
Khaoneow Damhmo^{B}	20.28^{a}	35.68±0.01^{b}	42.37±0.02^{b*}	28.21±0.05^{a}	36.02±0.03^{b*}	2.52±0.12^{e}	3.33±0.21^{b*}
Khaoneow Dam^{B}	19.25^{c}	29.65±0.01^{c}	35.42±0.01^{d*}	21.89±0.05^{b}	24.57±0.02^{g*}	2.64±0.29^{d}	3.31±0.39^{c*}
Khao Kam Doi^{P}	17.87^{g}	20.67±0.02^{f}	27.78±0.01^{f*}	15.55±0.03^{f}	26.47±0.03^{e*}	2.27±0.51^{i}	2.97±0.41^{e*}
Khow Kam^{P}	18.42^{e}	17.40±0.01^{j}	21.36±0.01^{k*}	15.50±0.07^{g}	16.47±0.03^{k*}	2.32±0.30^{h}	2.38±0.23^{i*}
Khaoneow Dang^{R}	15.52^{k}	15.60±0.01^{l}	19.50±0.01^{o*}	13.38±0.03^{i}	15.30±0.02^{n*}	1.24±0.21^{n}	1.32±0.31^{o*}
Non-glutinous rice							
Mali Nin rice^{P}	19.55^{a}	22.64±0.02^{e}	36.02±0.03^{c*}	20.21±0.11^{c}	28.03±0.03^{c*}	3.12±0.12^{a}	3.27±0.35^{d*}
Hom Nin rice^{P}	18.41^{e}	25.25±0.02^{d}	32.80±0.04^{e*}	17.75±0.07^{d}	27.67±0.08^{d*}	2.87±0.12^{b}	2.88±0.66^{g*}
Homnin-Jakkapat rice^{P}	19.23^{c}	20.27±0.44^{g}	23.72±0.02^{g*}	17.25±0.02^{e}	24.12±0.01^{h*}	2.38±0.23^{g}	2.94±0.21^{f*}
Rice Berry rice^{P}	20.28^{a}	18.74±0.02^{h}	22.84±0.02^{h*}	15.54±0.56^{f}	25.12±0.05^{f*}	2.22±0.12^{j}	2.95±0.71^{f*}
Hommali Dam rice^{B}	18.96^{d}	16.65±0.02^{k}	22.61±0.05^{i*}	14.40±0.05^{h}	24.61±0.02^{g*}	2.51±0.06^{e}	2.63±0.78^{h*}
Homdam Sutabut rice^{B}	17.96^{f}	18.20±0.05^{i}	21.94±0.01^{j*}	13.38±0.03^{i}	16.50±0.01^{k*}	1.57±0.12^{k}	1.82±0.34^{l*}
Hommali Dang rice ^{R}	15.82^{j}	15.50±0.02^{n}	21.03±0.02^{l*}	14.25±0.01^{h}	20.37±0.02^{i*}	1.32±0.21^{l}	1.37±0.56^{n*}
Sang Yod rice ^{R}	15.22^{m}	15.55±0.01^{m}	20.27±0.02^{n*}	13.33±0.63^{i}	18.03±0.02^{j*}	2.68±0.08^{c}	1.87±0.11^{k*}
Niang Guang rice ^{R}	17.48^{h}	14.44±0.01^{q}	20.57±0.01^{m*}	14.28±0.02^{h}	16.27±0.06^{l*}	1.26±0.21^{m}	1.45±0.36^{m*}
Tubtim Chumphae rice ^{R}	15.27^{l}	14.36±0.23^{p}	19.30±0.01^{p*}	12.28±0.05^{j}	15.44±0.02^{m*}	1.18±0.54^{o}	1.89±0.53^{j*}

Remark: B=black rice, P=purple rice, R= red rice

Mean values for each parameter followed by a different letter within each column are significantly different ($p \leq 0.05$) according to Duncan's Multiple Range test, * Means within each row between before and after fermentation are significantly different ($p \leq 0.05$) according to T-test.

the concentration of antioxidant that caused the decrease of DPPH radicals to half of its initial concentration. Therefore, the lower of EC_{50} value provides higher antioxidant efficiency. The antioxidative capacity of Khaow-Mak produced from colored rice was determined the free radical scavenging test using DPPH solution as present in Table 2. The EC_{50} values for the fermented black and purple rice varieties varied from 0.364 to 1.032 and 0.655 to 0.901 mg/ml respectively, while the fermented red rice varieties were in the range of 1.129-1.985 mg/ml.The lowest EC_{50} value was found in Leum Phua glutinous rice, corresponded to the highest content of TPC, TFC and TAC content that observed in this sample. Khaow-Mak produced from Leum Phua glutinous rice gave the strongest free radical scavenging activity with the EC_{50} value of 0.364±0.02 mg/ml. It was found that Khaow-Mak after fermentation had the strongest radical scavenging; higher than before fermentation. Moreover, the free radical scavenging activity from black rice and purple rice was found higher than red rice. Sangkitikomon et al. (2008) suggested that black rice's anthocyanin performs higher antioxidant activity than red rice and other rice varieties. In addition, total antioxidant capacity was determined by ferric reducing antioxidant power (FRAP). The FRAP activity in the phenolic extracts is related to the level of phenolic compounds. It is simple, fast and reproducible (Wong et al., 2006). It measures the ferric to ferrous reduction

in presence of antioxidants, which are effective as secondary antioxidants because they reduce the redox potential. It was noted that fermented colored rice had higher reducing abilities than that of unfermented rice of the same variety. The fermented black and purple rice varieties were not clearly differentiated in terms of FRAP values, whereas the lowest of FRAP value was observed from the fermented red rice varietie. The FRAP value of fermented black rice and purple rice was found higher than red rice with a range of 15.204-28.018, 14.136-20.588 and 7.948-14.667 mgFe(II)/g respectively.The fermented Leum Phua glutinous rice also presented the greatest FRAP value (28.018±0.01 mgFe(II)/g).

Table 2 Antioxidant performance of Khaow-Mak extracts from colored rice

Sample (Color rice varieties)	DPPH assay (EC_{50}) mg/ml		FRAP assay mgFe(II)/g	
	Before fermentation	After fermentaion	Before fermentaion	After fermentaion
Glutinous rice				
Leum Phua rice^{B}	1.215±0.06^{k}	0.364±0.02^{p*}	536.18±0.01^{e}	28.018±0.01^{a*}
Khaoneow Damhmo^{B}	1.362±0.01^{i}	0.432±0.03^{o*}	236.22±0.02^{a}	16.775±0.04^{f*}
Khaoneow Dam^{B}	1.236±0.02^{j}	0.502±0.02^{n*}	536.14±0.06^{i}	15.997±0.09^{g*}
Khao Kam Doi^{P}	1.563±0.02^{h}	0.858±0.05^{j*}	988.16±0.02^{f}	17.343±0.01^{e*}
Khow Kam^{P}	1.362±0.01^{i}	0.924±0.02^{g*}	633.11±0.02^{k}	14.136±0.05^{l*}
Khaoneow Dang^{R}	2.623±0.01^{b}	1.825±0.02^{c*}	858.6±0.02^{n}	10.582±0.02^{m*}
Non-glutinous rice				
Mali Nin rice^{P}	1.653±0.01^{g}	0.655±0.02^{m*}	19.869±0.02^{c}	19.127±0.02^{e*}
Hom Nin rice^{P}	0.956±0.07^{m}	0.852±0.02^{k*}	14.536±0.02^{i}	17.862±0.01^{d*}
Homnin-Jakkapat rice^{P}	0.985±0.01^{l}	0.842±0.01^{l*}	19.786±0.06^{d}	20.588±0.09^{b*}
Rice Berry rice^{P}	0.952±0.01^{n}	0.901±0.01^{h*}	20.436±0.05^{b}	15.895±0.01^{h*}
Hommali Dam rice^{B}	0.945±0.02^{o}	0.894±0.02^{i*}	15.697±0.01^{g}	15.424±0.02^{i*}
Homdam Sutabut rice^{B}	2.336±0.01^{e}	1.032±0.01^{f*}	15.063±0.05^{h}	15.204±0.09^{j*}
Hommali Dang rice^{R}	2.384±0.01^{d}	1.129±0.01^{e*}	9.763±0.05^{m}	14.667±0.02^{k*}
Sang Yod rice^{R}	2.653±0.01^{a}	1.635±0.02^{d*}	12.663±0.02^{j}	10.017±0.06^{n*}
Niang Guang rice^{R}	2.269±0.02^{f}	1.963±0.01^{b*}	5.368±0.08^{o}	8.497±0.06^{o*}
Tubtim Chumphae rice^{R}	2.542±0.25^{c}	1.985±0.01^{a*}	9.869±0.07^{l}	7.948±0.06^{p*}

Remark: B=black rice, P=purple rice R= red rice
Mean values for each parameter followed by a different letter within each column are significantly different ($p \leq 0.05$) according to Duncan's Multiple Range test, * Means within each row between before and after fermentation are significantly different ($p \leq 0.05$) according to T-test.

Our result agreed with Oki et al. (2002) who reported that anthocyanin from black rice and purple rice was found higher antioxidant activity than other pigmented rice. In addition, Researchers have demonstrated a positive correlation between the concentration of phenolic compounds and the antioxidant activity (Zhang et al., 2006). This might be due to the higher content of total phenolic compounds, anthocyanins and antioxidant activities in fermented rice probably because of the catalytic action of enzymes produced by the starter organisms in Look-Pang such as *S. cerevisiae, Aspergillus* spp. and *Rhizopus* spp. during fermentation which are capable of hydrolyzing glucosides of the inactive components to the active. Therefore, the action of enzyme such as beta-glucosidase produced by the starter organism during fermentation might be an important factor contributing to the increase of phenolic and anthocyanin contents of fermented rice (Plaitho et al., 2013). Anthocyanins are commonly a group of pigments found in pigmented rice such as purple, black and red rices. These compounds provide many biological properties such as scavenging free radicals.

Wang & Jiao (2000a) the researchers, reported that Thai pigmented rice such as black glutinous rice and Hom Mali Daeng had higher phenolic compounds, total flavonoid and antioxidant activity than normal white staple rice. Moreover, Pramai & Jiamyangyuen (2016) reported that total phenolic and flavonoid contents were the highest in the black rice followed by red rice and antioxidant capacities were predominant in pigmented varieties. Black rice grown in mountainous area presented the highest antioxidant activity compared to the other growing locations. From our experiment found that fermented Leum Phua glutinous rice had the highest contents of total phenolic, flavonoid, anthocyanin contents and showed the hightest antioxidant performance. It can be grown only once a year during rainy season in mountainous area and able to survive in high levels of water around Northern Thailand such as, Tak, Phitsanulok, Chiang Rai, and Phetchabun province. Luem Pua is one of the aromatic and indigenous black (dark purple) sticky rice, enriched with flavonoids, especially anthocyanins, and have total antioxidant higher than other black rices (Suwannalert & Rattanachitthawat, 2011; Wang & Shu, 2007; Boonsit et al., 2010). In experiment of Nakornriab, (2018) suggests that phenolic compounds are the major contributors to the antioxidant activities of brown rice. In addition, germinated brown rice is a potential source

of antioxidative and phytochemicals.

Glutinous rice differs from the non-glutinous rice mainly in having low (<5%) or almost no amylose in its starch but basically high in amylopectin. In general, the amylose content of rice starch varies from 0-2% in glutinous. Non-glutinous rice has higher amylose content but less sticky texture than glutinous rice (Setyaningsih et al., 2015). In this study, the level of total phenolics in glutinous rice was higher than its non-glutinous variety. The rate of enzymatic hydrolysis of the polymeric materials softens the rice kernels was fastest in glutinous rice because amylopectin content plays a major role in water hydration as it absorbs water faster than amylose. However, the level of phenolic compounds in different varieties of rice grains may diverge in phenolics concentrations. This discrepancy reveals that one would also expect changes due to the differences on their type of starch that have been distinguished as glutinous and non-glutinous variety (Mi-Young et al., 2010). In contrast, our result not agreed with Setyaningsih et al. (2015) who reported that the composition of phenolic compounds are noticeably different between glutinous and non-glutinous rice grains. The level of total phenolics in non-glutinous rice was higher than its glutinous variety. Hence, higher amylose content exhibits relatively higher amount of antioxidant compounds. The difference of phenolics content between black glutinous and black non-glutinous rice was not as impressive as if compared to the non-pigmented rice. Thus, in this particular case, phenolics concentration in rice appears to be strongly related to the pigment of rice in their bran. Both black pigmented glutinous and non-glutinous rice grains were produced without a bran removal process called polishing. Hence, as the phenolic compounds are mainly associated with the pericarp in the grain, the milling process to produce polished grain reduces the level of these compounds in the grain.

Conclusion

Various rice, including glutinous rice (black, purple and red) and non-glutinous rice (black, purple and red) were fermented for total phenolic, flavonoid, and anthocyanin contents analysis. The results showed that the content of bioactive compounds of all rice colors increased after fermention. Moreover, Khaow Mak produced from black and purple colored rice gave the content higher than red colored rice. The obtained results showed that each sample exhibited higher than that of the unfermented one of the same variety. However, Leum Phua glutinous rice had the highest contents of total phenolic (45.66±0.01 mgGAE/g), flavonoid contents (39.35±0.07 mgRE/g) and anthocyanin contents (3.37 ± 0.21 mg/g). In addition, Leum Phua glutinous rice showed the hightest antioxidant activity, including 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (EC_{50} 0.364±0.02 mg/ml) and ferric reducing antioxidant power (FRAP) (28.018±0.01 mgFe (II)/g).

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The Effect of Cooking Process and Shelf Life Evaluation of Retort Pouch Packed a Tradition Meat Curry "Kaoyuk"

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Abstract

Kaoyuk is a traditional meat curry of Trang province, Thailand. It is prepared as a standard recipe. A retort pouch is used because it is a convenient thermal process in which the texture and flavor should be maintained in something close to the original condition. The purpose of the research is to were investigate the effect of the pre-cooking time of Kaoyuk and the change in quality of retorted Kaoyuk after 12 months storage. The effect of the pre-cooking time of meat at 0, 60 and 120 min before retorted process were studied and the texture analyzed and a sensory evaluation undertaken. The result found that the pre-cooking time at 60 min provided the tenderest meat, the retorted Kaoyuk was softened and also more retained the sensory qualities of the original. The 60 min of pre-cooked Kaoyuk were packed in retort pouch with thermal process on F_0=11 min (118°C) and stored at room temperature (25±5°C) for 12 months. Physical properties, chemical properties, microbial properties and sensory evaluation were conducted every 3 months. Meanwhile, there was a significant decrease of pH from 5.9 to 5.46 and cutting force required was also was decreased from 8116 g force to 6320 g force whereas color L* a* b* values increased. The Free Fatty Acid value (FFA) was increased from 0.54 to 0.97 % of oleic acid and Thiobarbituric acid values (TBA) was increased from 0.64 to 1.07 mg of MDA/kg meat but no significant (P>0.05) growth of microbial and the sensory evaluation conducted from 10 trained panelists were accepted. The result showed that the retorted Kaoyuk can be store at room temperature for 12 months whilst maintaining acceptable safety and customer satisfaction levels.

Introduction

Traditional food is a central tenet of cultural heritage which links to the past of particular region. The food culture richness was presented with their ingredients and cooking methods (Kaban, 2013). Kaoyuk is a popular traditional meat curry of Trang province, which is in the southern part of Thailand. Kaoyuk is cultural food linked to celebrations and in daily life . It is often eaten at such festivals as Ching Ming festival, Chinese New Year festival and at wedding ceremonies. Kaoyuk

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is prepared from pork belly, and cooked with various spices in gravy and fried taro for 2 hours until the meats become soft, savory and crumbly. Therefore, it requires a long cooking time which is inconvenient and unsuitable for modern lifestyle. Furthermore, Kaoyuk is the product that people who visit home at Trang province and traveler buy to take back to their family. If the a form of Kaoyuk can be developed that can be stored for a longer period it will be convenient for the consumer and should increase sales volume for local products and local enterprise.

Retort food is convenient in terms of preparation via microwave or ready to eat because retort processing technology utilizes the thermal processing method, which is used for producing stable packed shelf products (Gokhale & Lele, 2014). Retort Pouch packaging material was developed using aluminum foil in different layers or a high barrier laminate package (Shah et al., 2017). Pouches are more familiar to and preferedby the customer than those packed in metal or glass due to them being lighter, more useful and more desirable for heating and preparation (Al-Baali & Farid, 2006). The product will be packed in high quality for energy server if use shorter retort time. Aside from these benefit, the products can be stored for a longer period at room temperature without refrigeration and chemical preservative (Shah et al., 2017).

However, the scientific data regarding the Kaoyuk in a retorted pouch is still scarce. Therefore, the aims of this study were to investigate the effect of pre-cooking time of Kaoyuk and evaluate retorted Kaoyuk shelf life during room temperature for 12 months.

Materials and methods

1. Materials

Pork belly, vegetables, spices and seasonings were purchased from the supermarket. Meat was brought to Food Laboratory of School of Culinary Arts, under refrigerated condition. Meat was boiled, deep fried and cut into pieces measuring 5x8.5x1 cm (W/L/D). Taro was cut in to pieces measuring 5x8.5x1 cm (W/L/D) and fried at 180°C for 5 min. The ingredients were then pre-cooked with gravy. The recipe formulation of Kaoyuk is given in Table 1. The details for producing Kaoyuk in a retort pouch are presented in Fig. 1.

Table 1 Recipe formulation of Kaoyuk

Ingredient	%	Ingredient	%
Belly pork	35.64	Chinese spice powder	0.36
Water	42.76	Chilli sauce	1.78
Salt	0.11	Pickled bean curd	1.46
Coriander root	0.25	Mono Sodium	0.14
Shalot	0.53	Glutamate	0.43
Garlic	0.53	Oyster sauce	0.18
Star anise	0.04	Soy sauce	0.14
Cinnamon	0.11	Palm sugar	0.39
Ginger	0.53	Sugar cane	14.25
Seasoning	0.36	Taro	

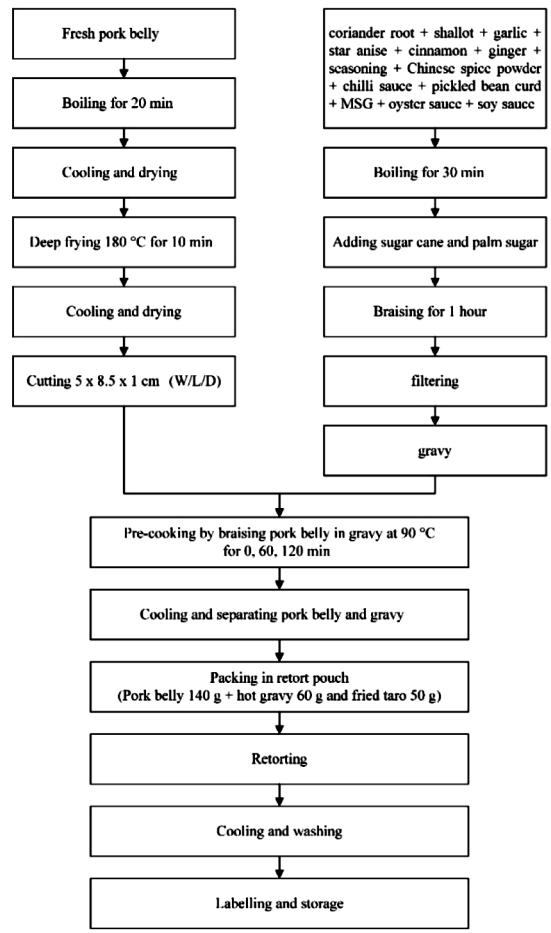

Fig. 1 The process of Kaoyuk in retort pouch

2. Packing and processing

The multilayer laminate retort pouch of dimension 132 x 200 x 28 mm. was used to pack the product. The pre-cooked meat was packed into the retort pouch manually (250 g of product: 140 g pre-cooked meat 50 g fried taro and 60 g gravy) then, a vacuum removed the

air before sealing the pouch using a sealer (Model: VM203, Audionvac). The packed and sealed pouches were carried on trays and moved into a retort vessel then sterilized in a retort (Model: A091 Fabric No. M4665 at 118°C 0.15 MPa with the F_0 = 11 mins.

3. Study of pre-cooking time

Pre-cooking time affected the texture of retorted Kaoyuk, this research needs to retain the quality of retorted Kaoyuk the same as the original unretorted food. Therefore, this study investigated three pre-cooking times; (at 90 ±5°C) 0, 60 and 120 min. The texture of meat was determined by texture analyzer and sensory evaluation was conducted by 10 trained panelists.

4. The quality change of Retorted Kaoyuk during storage

Retorted Kaoyuk which best pre-cooking time, were stored at room temperature (25±5°C) for 12 months. The retorted Kaoyuk were evaluated every 3 months by taking the meat sample to measure physical properties, chemical properties, microbial properties and sensory analysis.

4.1 Physical Properties

The color measurement of meat was determined using Hunter Lab Color Flex (Model A60-1012-312, Hunter Associates Laboratory Inc., USA). CIE L* (Lightness) a* (redness) and b* (yellowness) were measure in the surface of samples.

The texture of belly meat was determined by texture analyzer (Model: TA-XTPlus) Blade Set. Cutting Strenght was recorded in gram force.

4.2 Chemical Properties

The meat was determined pH by adding 10 g sample with 50 ml distilled water and mix for 60 s in a mixer. The pH values were determined using a digital pH meter (Mettler toledo Model: Seven Compact pH meter, S210-Bio, Switzerland), Calibrated using pH buffer 4 and pH buffer 7.

The meat, taro and gravy were determined for moisture, crude protein, crude fat and total ash with the following of standard procedures (AOAC., 1995). The carbohydrates were calculated by subtracting from 100 the percentages of crude protein, moisture, crude fat and total ash.

The Free Fatty acid (FFA) content (as Oleic acid) using titrimetric method follow AOAC 940.28 (AOAC., 2000).

The lipid oxidation of meat was analyzed by measuring Thiobarbituric acid (TBA) using method of Egan et al. (1981). Result of TBA expressed as mg of malondialdehyde (MDA)/kg of sample.

4.3 Microbial properties

Retorted Kaoyuk were analyzed from its commercial ready to eat product. The sample was determined for total viable count, *E. coli* and Coliform, Yeast and Mold count, *Staphylococcus aureus* and *Salmonella* spp. followed BAM (2001).

4.4 Sensory Analysis

The sensory evaluation of Kaoyuk was conducted from 10 trained members from School of Culinary Arts according to American Meat Science Association Guideline (AMSA, 2015). The Kaoyuk was evaluated on appearance, flavor, texture, tenderness, juiciness and overall acceptability on a 9-point scale (where, 1 = dislike extremely, 9 = like extremely). The samples were warmed before serving. The samples were in white cups coded with 3-digit numbers and served to the panelists individually in a random order.

5. Statistical analysis

The statistical analysis was done by SPSS software (SPSS Version 17; SPSS Inc., Chicago, USA). Data were analyzed by ANOVA and the means were separated using Duncan's New Multiple Range Test. The statistical significance was determined at 95% confidence level ($P<0.05$).

Results and discussion

1. Study of pre-cooking time

The meat was pre-cooked for 0, 60 and 120 min before packed and thermal processing. The texture of pre-cooked meat for 0, 60 and 120 min were 13072, 8191 and 10512 g force. These were related to panelist's sensory evaluation in Table 2 that the pre-cooked meat at 0 and 120 min required a high cutting force and were unacceptable in their texture. Pre-cooked meat at 0 min required a higher cutting force because the time made the collagen fiber shrinkage (Tornberg, 2005). A longer cooking time makes collagen soluble which causes tenderness (Hedruck et al., 1994; Shah et al., 2017). Retorted Kaoyuk was curry meat contain sugar in the gravy, if take too long heating time the meat will drip loss and replaced with sugar which makes the meat hard. Therefore, the pre-cooked Kaoyuk at 120 min was considered too tough by the sensory evaluations of the panelists. As a result, pre-cooked Kaoyuk at 60 min required a lower cutting force the same as the original and the panelists found it the most acceptable of those sampled.

Table 2 Sensory evaluation of pre-cooked Kaoyuk

Characteristics	Pre-cooking time (min)		
	0	60	120
Appearance	4.2 ± 0.79[c]	6.7 ± 0.95[a]	5.2 ± 0.79[b]
Flavor	3.1 ± 0.74[c]	6.2 ± 0.79[a]	5.5 ± 0.85[a]
Texture	3.2 ± 0.79[c]	6.3 ± 0.48[a]	4.3 ± 0.16[b]
Tenderness	2.5 ± 0.53[c]	6.5 ± 0.71[a]	5.4 ± 0.52[b]
Juiciness	3.2 ± 0.40[c]	7.1 ± 0.83[a]	5.7 ± 0.88[a]
Overall acceptance	2.4 ± 0.70[c]	6.5 ± 0.53[a]	5.5 ± 0.88[b]

Remark: [a-c] superscripts in the same row indicate significant difference (P<0.05).

2. The quality change of retorted Kaoyuk during storage

The retorted Kaoyuk which pre-cooking for 60 min was storage at room temperature for 12 months, then there were evaluated every 3 months to measure physical properties, chemical properties, microbial properties and sensory evaluation

Table 3 Physical properties of retorted Kaoyuk during storage at room temperature

Time (month)	Cutting force (g force)	color		
		L*	a*	b*
0	8116±118[a]	53.41±1.97[a]	14.083±0.39[c]	28.692±0.63[c]
3	7886±228[ab]	47.37 ±0.74[b]	19.65±0.57[d]	30.85±1.34[d]
6	7558±236[b]	41.29±1.02[c]	21.15±0.47[c]	32.82±0.97[c]
9	7056±150[c]	38.95±0.79[d]	24.38±0.57[b]	35.23±0.35[b]
12	6320±205[d]	35.23±0.91[e]	26.31±0.89[a]	38.48±0.55[a]

Remark: [a-e] superscripts in the same column indicate significant difference (P<0.05).

The texture of retorted Kaoyuk was determined as cutting force and recorded in gram force. Cutting force was significantly different for different storage time. From Table 3, the cutting force was decreased with increased storage time, this condition shown that fibrous protein was broken by heating. Furthermore, protein oxidation is the cause of the shear force value decreasing when increasing meat storage time (Devadason et al., 2014).

The color data like L*(darkness to lightness), a* (redness to green) and b* (blue to yellowness) of retorted Kaoyuk which stored at 0-12 months as given in Table 3. There was significantly decreased in L* value whereas increased in a* and b* value when increased storage time. Millard reaction between amino acid and sugar will be the cause of color changing (Bindu et al., 2007)

The proximate composition of retorted Kaoyuk were 57.1% of moisture, 10.6 of protein, 11.5 % of fat, 17.3 of Carbohydrate, 2% of ash, 1.5 % of fiber and 215 kcal of energy.

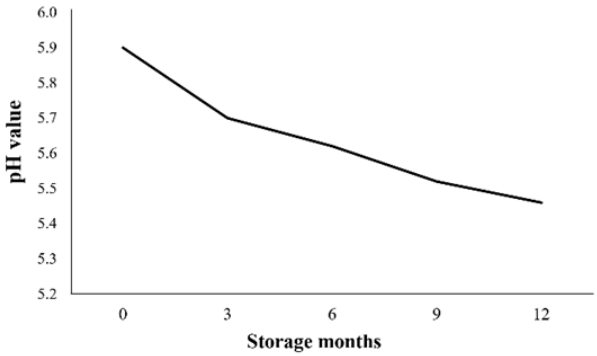

Fig. 2 The pH value change during storage time of retorted Kaoyuk

During storage, pH value of retorted Kaoyuk was decreased from 5.90 to 5.46 in 12th month (Fig. 2). The decreasing of pH is due to protein degradation and free amino acids being released. (Devadason et al., 2014). A decrease of pH value has also been observed during the storage buffalo meat blocks (Devadason et al., 2014) and retorted beef curry (Tayeh et al., 2019). Some researchers reported that the lipid oxidation is the cause pH reduction by residue oxygen because of carbohydrate or glucose of lipid components were degradation (Jang & Lee, 2012; Liu et al., 2009; Jin et al., 2002).

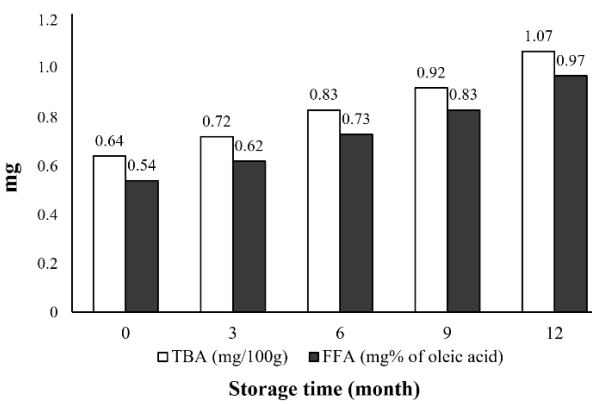

Fig. 3 Free fatty acid value and TBA value of retorted Kaoyuk

The free fatty acid (FFA) values of retorted Kaoyuk are presented in Fig. 3. Free fatty acid is the product of lipid hydrolysis, retorted Kaoyuk were in high temperature process. Therefore, the free fatty acids were generated in the high temperature. FFA values were increased significantly with increased storage time and the highest being reported in the 12th month. Similar trends were reported for retorted black clam (Bindu et

al., 2007) and retorted meat curry (Shah et al., 2017).

TBA value is a chemical spoilage indicator of meat and meat product. Lipid oxidation is the cause of TBA value, produce free radical and generate rancid flavors and odors. TBA value of retorted Kaoyuk was presented in Fig.3. Retorted Kaoyuk increased in TBA value from 0.64 - 1.07 mg malonaldehyde per kg of meat with an increase of storage time from 0^{th} - 12^{th} month as same as the TBA trend of retorted buffalo meat, (Devadason et al., 2014) retorted black clam meat curry (Bindu et al., 2007) and retorted meat curry (Shah et al., 2017).

Retorted Kaoyuk were analyzed for total viable count, *E.coli* and Coliform, Yeast and Mold, *Staphylococcus aureus, Salmonella* spp. presented in Table 4. There are less than 10 CFU/g of total viable count and *E.coli* and Coliform. There are less than 3 CFU/g of Yeast and Mold and not found *Staphylococcus aureus* and *Salmonella* spp. for 3-12 months. It indicated that microbial in retorted Kaoyuk were destroyed by thermal processing as the retorted tradition meat curry was undetected during storage for 12 months (Shah et al., 2017). Moreover, Rajan et al. (2014) reported that there were undetected growth of microbial in retorted Chettinad chicken packed in pouch during storage for 180 days.

Table 4 The microbial properties of retorted Kaoyuk during storage at room temperature (25 ±5 °C)

microbial	storage time (month)				
	0	3	6	9	12
Total viable count (CFU/g)	<10	<10	<10	<10	<10
E. coli & Coliform (MPN/g)	<10	<10	<10	<10	<10
Yeast and Mold (CFU/g)	<3	<3	<3	<3	<3
Staphylococcus aureus (CFU/g)	ND	ND	ND	ND	ND
Salmonella spp. (CFU/g)	ND	ND	ND	ND	ND

Remark: ND = not detect

When increasing the storage time to 12 months, the cutting force, color (L*) and sensory score of retorted Kaoyuk were decreased and the FFA, TBA were increased. It indicated that the quality of retorted Kaoyuk was changed during storage at room temperature because of their physical and chemical properties. However, the panelists were accepted in their sensory evaluation at the 12^{th} month of storage time as shown in Table 5. The scores for each parameter were significantly decreased with increased storage time. Although the score of appearance, flavor, texture, tenderness, juiciness and overall acceptance decreased to 5.6 hence panelists found 12 months retorted Kaoyuk was acceptable. If the retorted Kaoyuk were kept further for 18 months, it will be safe from microorganism but it may contain more rancid and become more unflavored.

Table 5 Sensory evaluation of retorted Kaoyuk during storage at room temperature

Characteristics	Storage period (month)				
	0	3	6	9	12
Appearance	8.0 ± 0.47^{a}	7.3 ± 0.48^{b}	7.1 ± 0.32^{b}	6.7 ± 0.48^{c}	6.6 ± 0.52^{c}
Flavor	8.1 ± 0.32^{a}	7.4 ± 0.52^{b}	6.4 ± 0.52^{c}	6.2 ± 0.42^{c}	6.1 ± 0.57^{c}
Texture	8.7 ± 0.48^{a}	7.9 ± 0.32^{b}	6.3 ± 0.48^{c}	6.2 ± 0.42^{c}	6.1 ± 0.57^{c}
Tenderness	8.4 ± 0.52^{a}	7.5 ± 0.53^{b}	6.6 ± 0.52^{c}	6.4 ± 0.52^{c}	6.3 ± 0.68^{c}
Juiciness	8.6 ± 0.52^{a}	7.7 ± 0.48^{b}	6.7 ± 0.48^{c}	6.3 ± 0.48^{c}	5.6 ± 0.51^{d}
Overall acceptance	8.5 ± 0.53^{a}	7.6 ± 0.52^{b}	6.4 ± 0.52^{c}	6.1 ± 0.32^{cd}	5.9 ± 0.32^{d}

Remark: $^{a-d}$ superscripts in the same row indicate significant difference ($P<0.05$).

Conclusion

The growth of demand for ready to eat meat curry product is due to several advantages. Kaoyuk, the traditional meat curry of Trang province was developed, packed and processed in retort pouch which can be store for 12 months. The studies included 2 parts, the first was the pre-cooking time which retain the retorted Kaoyuk same as original and the second was the investigation into any quality change of retorted Kaoyuk when kept in room temperature for 12 months. The result found that the pre-cooking time at 60 min is the best condition of Kaoyuk producing before sterilized with retort process. Retorted Kaoyuk was evaluate the physical properties, chemical properties and sensory evaluation in every 3 months which found that pH value and cutting force were significantly decreased whereas color value (L*), FFA and TBA value increase with the increase of storage time to 12 months. In addition Retorted Kaoyuk was safe from microorganism during storage at room temperature for 12 months, although the sensory evaluation had decreased the score but it was still within acceptable limits. Therefore, Kaoyuk has accepted a product that meeting customer need with extending shelf life on display.

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Assessment of Carbon Dioxide Captured in Producer Biomass and Its Influencing Factors in a Tropical Freshwater Reservoir

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Abstract

Since the impact of global warming and climate change due to emissions of greenhouse gas is increasingly serious, freshwater aquatic ecosystems are considered to be one of the most important natural carbon sinks. The key process of the carbon cycle and energy flow in the system is photosynthesis where CO_2 is fixed to produce organic compounds by aquatic producers or phytoplankton. The concept of net primary productivity (NPP) is generally used to describe the net amount of energy and CO_2 stored in producer biomass. Nonetheless, phytoplankton primary production depends directly on various physicochemical as well as biological factors. This study investigated the variation of NPP to estimate CO_2 absorption in relation to the influence of physicochemical parameters in tropical freshwater ecosystems by using Srakaew reservoir as a case study. Water samples were collected in three consecutive seasons during September 2018 to April 2019. The results revealed that CO_2 captured by phytoplankton in the reservoir to produce their biomass or NPP range from 350 to 5,777 mg m^{-2} day^{-1} (mean = 2,813 mg m^{-2} day^{-1}). CO_2 absorption displayed a significant linear relationship with light intensity and water temperature. Seasonal variation can affect NPP and CO_2 absorption. In the hot season, NPP and CO_2 absorption in the water were significantly higher than the cool season while there was no significant difference in the rainy season compared with the other seasons. According to the trophic state assessment, Srakaew reservoir was classified as eutrophic and hypereutrophic due to its low Secchi transparency coupled with high nutrient levels in the water.

Introduction

The threat of global warming and climate change is increasingly seen as one of if not the primary international environmental concern. The phenomenon is attributed to an increase of greenhouse gas (GHG) emissions to the atmosphere of which carbon dioxide (CO_2) contributes over 65% of total GHG emissions (IPCC, 2014). As the situation of the impact is increasingly severe, it is necessary to seek an appropriate methodology to reduce the emissions and to accumulate CO_2. There are two main approaches of CO_2 sequestration

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globally adopted, namely physical and biological strategies (Khoo et al., 2011). Nonetheless, due to a number of limitations associated with physical techniques, technical improvements are still under investigation (Bhola et al., 2014). On the other hand, biological CO_2 capture appears to be a more promising technique since the method is normally a part of natural processes in ecosystems and less complicated than the physical techniques (Kumar et al., 2010). The key process is CO_2 fixation during photosynthesis to produce organic compounds by producers including plants and other photosynthetic organisms. Therefore, ecosystems, both terrestrial and aquatic, are considered to be the largest natural carbon sinks.

The concept of primary productivity is normally used to describe the amount of energy and CO_2 fixed by producers. It is also used as a biodiversity index of an ecosystem that is directly or indirectly controlled by the biotic and abiotic factors (Paul et al., 2006). Primary productivity is the rate at which solar energy is converted into chemical energy by photosynthetic (or chemosynthetic) autotrophs in an ecosystem while the accumulation of energy in producers called primary production (Miller & Spoolman, 2009). Primary productivity can be categorized into Gross Primary Productivity (GPP) and Net Primary Productivity (NPP). GPP refers to the total amount of chemical energy produced in the process, whereas NPP is the remaining chemical energy from aerobic respiration of producers and stored in their tissue (biomass). Thus, NPP can be used as an indicator of the capacity of an ecosystem to accumulate carbon (Lin, 2014). There is evidence that changes in NPP directly influence the amount of carbon stored in ecosystems (Reich et al., 2001). In terrestrial ecosystems, plants as producers can sequester a large amount of CO_2 as biomass from the atmosphere. However, previous studies indicated that aquatic producers such as microalgae and cyanobacteria have more rapid growth rates and more efficient CO_2 fixation than terrestrial plants (Costa et al., 2000; Langley et al., 2012). In addition, Sydney (2010) suggested that approximately 513 ton of CO_2 per ha per year can be sequestered in aquatic ecosystems during the process of biomass production of phytoplankton. Studying on primary productivity of producers and its influencing factors in aquatic ecosystems is therefore beneficial to understand natural mechanisms of carbon sequestration.

In aquatic ecosystems, the processes of carbon assimilation expressed as primary productivity of producers display complex relationships where solar energy is converted to chemical energy under the influence of other physicochemical factors in water. Light and nutrients are frequently found to be the main limiting factors in freshwater lakes (Guildford & Hecky, 2000; Simmons et al., 2004). Light availability is a factor that determines the vertical distribution of photosynthetic autotrophs and their production rates (Tonetta et al., 2015). Moreover, seasonal variations of light intensity and temperature can also influence on the distribution of phytoplankton (Vaillancourt et al., 2003). In the tropical zone where weather conditions have no obvious pattern like in the temperate zone, weather conditions such as rain and cloud cover can disrupt the intensity of light, temperature and physicochemical properties of water that consequently affect the process of photosynthesis of aquatic producers (Guildford et al., 2007; Omar et al., 2016).

Phytoplankton productivity is sensitive to nutrient availability, particularly phosphorus. In oligotrophic (low-productivity) lakes, the growth of phytoplankton and production are usually constrained by phosphorus limitation (Liboriussen & Jeppesen, 2003). Pollutants from anthropogenic activities such as nutrient leaching from fertilizers, release of washing detergents and other domestic wastes can induce an imbalance of nutrient in water leading to eutrophication (Chaudhuri et al., 2012). Apart from physicochemical factors, lake trophic state or a measure of the productivity of an aquatic ecosystem can be used to describe the potential of lake for primary production. Carlson & Simpson (1996) suggested a production-based trophic state index (TSI) using three index variables (water transparency, total phosphorus and/or chlorophyll a) that are interrelated by linear regression models and should lead to the same index value for a given combination of variable values. As such, any of the three variables can theoretically be used to classify a lake or reservoir. Four states of lake are classified based on TSI value including oligotrophic (low-productivity), mesotrophic (moderate-productivity), eutrophic (high-productivity) and hypereutrophic (very high-productivity) (Carlson & Simpson, 1996). Yet, the state of a freshwater lake can be varied from time to time as a result of changes in temporal and spatial factors (Kuehl & Troelstrup, 2013).

Besides natural aquatic ecosystems, artificial or man-made aquatic ecosystems are likely to be important in terms of the ecological and socio-economic value in urban areas; they play an equivalent role in providing

ecosystem services including carbon sequestration (Clifford & Heffernan, 2018). In this study, Srakaew reservoir that is an artificial aquatic ecosystem located in the urban area of Nakhon Pathom province was used as a case study. The reservoir is considered to be a good example of small-scale artificial aquatic ecosystems in urban areas. Additionally, the site can represent a lake that is affected by human activities in both rural and urban communities as it is supplied with a canal flowing from agricultural lands. The study site is situated in Silpakorn University surrounded by urban communities that may discharge pollutants and nutrients into the water. The objectives of this study were to (1) estimate NPP and the trophic state of tropical freshwater ecosystems, (2) assess the potential of CO_2 absorption by calculating CO_2 captured by aquatic producers to produce their biomass or NPP and (3) examine the relationship between CO_2 absorbed in producer biomass and associated physicochemical parameters including water temperature, Secchi transparency, light intensity and total phosphorus.

Materials and Methods

1. Site description

The study was conducted in Sakaew, a small man-made reservoir situated in Silpakorn university Sanamchandra palace campus, that is located at the central areas of Nakhon Pathom province, Thailand (latitude 13°48' to 13°49' N; longitude 100°02' to 100°03' E) (Fig. 1). The reservoir covering a total area of 17,700 m^2 with mean depth of 3 m was constructed in 1903; at the same time that Sanamchandra palace was built in the reign of king Rama VI. It is currently used for recreational purposes, fish species conservation and as a shelter for some migratory birds. The reservoir is supplied with water by rainfall as well as from Chedi Bucha canal flowing from the north-west of the province through community areas and agricultural lands before filling the reservoir and then running eastward to meet the Tha Chin River at Nakhon Chaisi district. The reservoir is currently under control of the university; and fishing activity is not allowed. No floating plants exist in the area. Emergent plants in the littoral zone are sparse and, therefore, not taken into account in this study. It is noted that the period of the hot (pre-monsoon) season of Thailand is normally from March to May, June to October for the rainy (monsoon) season and November to February for the cool (post-monsoon) season (TMD, 2018).

2. Field measurements

A 7-month study was undertaken during the period of September 2018 to April 2019 to assess net primary productivity and related physicochemical parameters. Five points of sample collection were defined across the reservoir as depicted in Fig. 1. Samples were collected once a week between 11.00 am and 02.00 pm. Secchi transparency, which determines the depth of photic zone, was measured at each point of collection by a Secchi disk. Average solar radiation of the day was recorded by an ambient air monitoring station, AQM model 65 (Aeroqual, Auckland, NZ), located adjacent to the collection points as exhibited in Fig. 1. Dissolved oxygen (DO) before and after incubation as well as water temperature were measured in situ by YSI model 54A dissolved oxygen meter (Yellow Spring Instruments, Ohio, US).

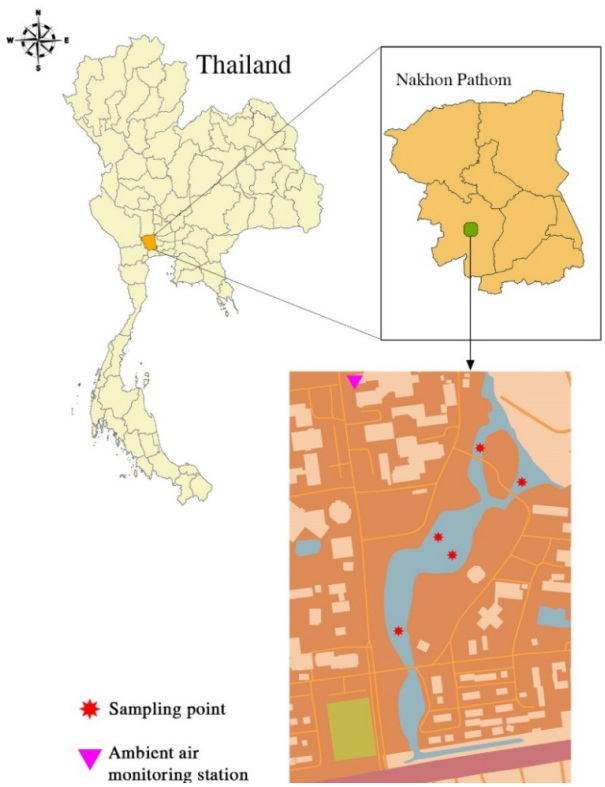

Fig. 1 Map of the study area: Srakaew reservoir, Nakhon Pathom, Thailand

3. Laboratory analysis

Water samples for total phosphorus analysis were collected from each point each testing day at the midpoint of the Secchi depth. The samples were subsequently combined following the spatial composite sampling method. The method accounts for horizontal spatial heterogeneity that provides an estimate of average water quality (Alberta Environment, 2006). Then, the water samples were brought to the laboratory for the analysis. Total phosphorus was determined by the ascorbic acid method following APHA, AWWA and WEF (2012) (Rice et al., 2012).

4. Measurement of primary production

Net primary production (NPP) was estimated according to the light/dark bottle method (Vollenweider, 1969). In the measurement, the decline of oxygen in the dark bottle that has been incubated for a period of time reflects the amount of respiration by consumers and producers whereas the oxygen change in the light bottle indicates the net result of oxygen produced and oxygen utilized by respiration. However, as the current study was focused on estimating NPP, only light bottles were used. Water samples were collected at the midpoint of the Secchi depth. An initial value of dissolved oxygen (DO) of light bottles was measured prior to the bottles being sealed and suspended at the midpoint of the Secchi depth and incubated for three hours (from 11.00 am to 02.00 pm). After the incubation, the bottles were collected and final DO concentrations were measured. NPP was estimated by dividing the change in dissolved oxygen (final-initial) by the incubation period and then multiplying by the number of daylight hours of each testing day. Nevertheless, due to lack of data, the number of daylight hours in this study was assumed to be 12 hours. In addition, as Thailand is located close to the equator, daylight hours are likely to be less variable compared to those countries located in temperate zone. The unit of NPP was converted from per volume to per area by multiplying the value by the Secchi depth. Finally, daily NPP was identified in carbon units per square meter following the unit conversion that 1 mg C is equal to 2.67 mg O_2 (Lind, 1985). The calculation is explained in equation (1).

In terms of CO_2 absorption, the net use of CO_2 by producers for biomass production can also be converted from NPP since 1 mgC of NPP is produced from 3.67 mg CO_2 according to the photosynthesis equation.

5. Trophic state index

Trophic state is a measurement of the productivity of a water body associated with correlated criteria. The trophic state of the reservoir was assessed by the Carlson Trophic State Index (TSI) considering measurements of Secchi transparency, total phosphorus, and/or chlorophyll a by applying the equations below (Carlson & Simpson, 1996):

$$\text{TSI Total Phosphorus} = 14.42 * \ln(\text{TP}, \mu g\ l^{-1}) + 4.15 \quad (2)$$

$$\text{TSI Secchi transparency} = 60 - 14.41 * \ln(\text{Secchi transparency, m}) \quad (3)$$

$$\text{TSI Chlorophyll a} = 9.81 * \ln(\text{chlorophyll a}, \mu g\ l^{-1}) + 30.6 \quad (4)$$

However, only two types of TSI calculated from total phosphorus or TSI (TP) and transparency or TSI (SD) were taken into account in this study. The Trophic State Index ranges from 0 to 100. Different index ranges indicate one of the following trophic categories: oligotrophy (less than 30), mesotrophy (30 to 49), eutrophy (50 to 70) and hypereutrophy (more than 70). It is noted that TSI calculated from different parameters are considered separately (not in average value) as individual surrogate indicators (Kuehl & Troelstrup, 2013).

6. Statistical analysis

The results of physicochemical parameters and NPP of each day of study represented an average of 5 samples collected from 5 points of the reservoir. Comparison of parameters in different seasons was analyzed using one-way ANOVA and multi-pairwise comparisons by Tukey's Honestly Significant Difference (HSD) post hoc test under 5% significance level. In addition, linear regression with $\log_e$ transformations was conducted in order to evaluate relationships of the variables by using the Statistical Package for the Social Sciences (SPSS, version 24) software. The results were expressed as differences between the groups considered statistically significant at $p < 0.05$ and highly significant at $p < 0.01$.

$$\text{NPP (mg C. m}^{-2}\text{.day}^{-1}) = \frac{\Delta\text{DO (mg. l}^{-1}\text{ day}^{-1}) * 1000\text{ (l. m}^{-3}) * \text{Secchi depth (m)}}{2.67\text{ mg O}_2\text{. mg C}^{-1}} \quad (1)$$

Results and discussion

1. Physicochemical parameters

Average water temperature of the reservoir throughout the period of study was 28.1°C. The lowest temperature was recorded in the winter month of January (23.9°C) and the highest temperature was found in March (32.3°C) demonstrated in Table 1. Table 2 shows seasonal average values of different parameters indicating that the average temperature and light intensity in the hot season was significantly different from those in the rainy and cool seasons. Light intensity highly depends on weather conditions, particularly cloud cover, in each day. In a cloudy, rainy day such as on October 26, average light intensity was recorded as low as 53.2 W. m^{-2}; whereas in a sunny day of summer on March 29, it reached 225.8 W. m^{-2}. The light penetration depth or transparency measured by Secchi disk is likely to be varied from day to day ranging from 24.3 cm to 34.3 cm. However, the transparency tends to be high from the end of rainy season (October) until the end of winter (February) with an average of 28.8 cm when the value tends to be low in the early summer from March onwards with an average of 25.8 cm. Total phosphorus concentrations ranged between 90 to 280 µg. l^{-1}. Although there is no significant difference influenced by seasonal variation, the overall trend of TP concentration was relatively high in the middle of the cool season in December. It can be as a result of high precipitation in the rainy season leading to elevating reservoir water volume and diluting TP in the water body (Ling et al., 2017).

2. NPP and CO_2 absorption

Phytoplankton biomass in the form of NPP within Srakaew reservoir reflected seasonal variation since there was a statistical difference between groups determined by one-way ANOVA ($F(2,22) = 8.487$, $p < 0.01$). As shown in Table 2, the results from Tukey post hoc test revealed that NPP in the hot season was statistically significantly higher than that in the cool season while no significant difference was observed for NPP in the rainy season compared with the other seasons. Seasonal trends in NPP follow the pattern reported in other tropical lakes located in the monsoon-influenced countries that NPP tends to be low in the monsoon and cool seasons and highest in the hot season (Chaudhuri et al., 2012; Sontakke & Mokashe, 2014; Kumar, 2015). The high NPP of lakes in the monsoon-influence zone in the hot season could be due to greater light intensity coupled with high temperature that increase photosynthetic production of phytoplankton, while the low in the monsoon and cool seasons could be due to low temperature and poor light intensity (Singh et al., 2018). The average NPP throughout the collecting period was 767 mgC. m^{-2}. day^{-1} that ranged from 96 to 1,576 mgC. m^{-2}. day^{-1}. Compared the results to previous studies, it appears that NPP of Srakaew reservoir showed a higher value than temperate lakes such as in Sweden (5-192 mgC. m^{-2}.day^{-1}) (Ask et al., 2009) and in South Dakota, USA (average 245 mgC. m^{-2}.day^{-1}) (Kuehl & Troelstrup, 2013). As for the other lakes located in the same climate zone, NPP of a lake in Assam varied from 110–1,750 mgC m^{-2}.day^{-1} (Sugunan & Bhattacharjya, 2000). Mean NPP of a lake located in Brahmaputra floodplain was 548 ± 87 mgC. m^{-2}. day^{-1} (Baruah, 2003). Moreover, compared with Tonle Sap, a large-scale tropical lake located in Cambodia, the range of NPP is likely to be approximate to the current study, which is 1,700–2,400 mgC. m^{-2}. day^{-1} (Holtgrieve et al., 2013). However, in some tropical eutrophic lakes such as Patna Pond in India where nutrients and light are abundant, the value of NPP can be as high as 5,469–9,964 mgC. m^{-2}.day^{-1} (Verma & Srivastava, 2016).

Table 1 Mean value of physicochemical parameters and NPP in Srakaew reservoir recorded in each collecting date

Date	Temperature (°C)	Transparency (cm)	Light intensity (W. m^{-2})	Total P (µg/l)	NPP (mg C. m^{-2}. day^{-1})
20-Sep	27.7 ± 0.3	27.2 ± 1.5	53.2	175	202 ± 210
28-Sep	29.0 ± 0.6	32.0 ± 1.0	166.3	280	1,047 ± 250
08-Oct	28.0 ± 0.5	25.6 ± 4.3	166.9	140	1,025 ± 374
15-Oct	28.2 ± 0.6	28.0 ± 2.9	157.2	130	1,333 ± 541
22-Oct	27.5 ± 0.5	29.9 ± 5.6	119.2	90	665 ± 231
29-Oct	27.6 ± 0.4	29.3 ± 6.4	112.3	90	547 ± 231
05-Nov	27.8 ± 0.4	25.9 ± 2.6	86.8	145	323 ± 131
17-Nov	28.9 ± 0.3	27.0 ± 3.8	135.5	160	802 ± 150
22-Nov	27.8 ± 0.7	28.0 ± 1.8	70.1	175	258 ± 184
27-Nov	27.4 ± 0.5	29.5 ± 3.3	141.2	150	785 ± 131
06-Dec	27.1 ± 0.4	29.3 ± 3.1	109.0	260	532 ± 164
13-Dec	25.8 ± 0.4	28.3 ± 2.3	138.0	240	346 ± 46
20-Dec	25.7 ± 0.6	25.6 ± 2.7	133.5	240	374 ± 143
04-Jan	23.9 ± 0.2	29.6 ± 4.5	75.9	190	193 ± 135
11-Jan	26.3 ± 0.4	27.6 ± 4.4	105.7	130	96 ± 48
18-Jan	25.9 ± 0.1	33.1 ± 1.7	88.8	155	272 ± 218
08-Feb	26.7 ± 0.5	27.0 ± 2.6	143.0	110	748 ± 147
15-Feb	29.6 ± 1.0	30.0 ± 1.7	125.7	200	1,427 ± 515
22-Feb	28.7 ± 0.6	34.3 ± 0.9	173.9	180	745 ± 306
01-Mar	31.0 ± 1.0	28.3 ± 2.0	164.2	180	1,156 ± 452
15-Mar	32.3 ± 0.4	24.3 ± 3.3	177.4	185	840 ± 277
22-Mar	30.0 ± 0.6	26.0 ± 2.8	189.2	185	1,341 ± 351
29-Mar	30.0 ± 0.5	25.2 ± 3.2	225.8	165	1,576 ± 94
05-Apr	30.1 ± 0.3	25.1 ± 1.9	222.7	205	1,337 ± 166
10-Apr	30.5 ± 0.6	25.6 ± 2.1	218.6	165	1,212 ± 74
Average	**28.2 ± 1.9**	**28.1 ± 2.5**	**140.0 ± 47.1**	**173.0 ± 46.9**	**767 ± 448**

Table 2 Seasonal variation of physicochemical parameters and NPP in Srakaew reservoir

Parameter	Unit	Rainy season[1]	Cool season[2]	Hot season[3]
No. of sample (n)		6	13	6
Temperature	°C	28.0 ± 0.5[a]	27.1 ± 1.6[a]	30.7 ± 0.9[b]
Transparency	cm	28.7 ± 2.2[ab]	28.9 ± 2.6[a]	25.8 ± 1.4[b]
TP	µg l^{-1}	150.0 ± 71.0[a]	179.6 ± 45.3[a]	180.8 ± 15.0[a]
Light intensity	W m^{-2}	129.2 ± 44.1[a]	117.5 ± 30.8[a]	199.7 ± 26.2[b]
NPP	mgC. $m.^{-2}$ day^{-1}	803 ± 409[ab]	530 ± 363[a]	1,244 ± 245[b]

Remark: [a-c] different letters in the same row represent statistical differences ($p<0.05$, Tukey's HSD test).

[1] data collection period: September 20, 2018 to October 29, 2018.

[2] data collection period: November 5, 2018 to February 22, 2019.

[3] data collection period: March 1, 2019 to April 12, 2019.

As far as CO_2 absorption is concerned, the amount of CO_2 absorbed in phytoplankton biomass is directly positively related to NPP and thus, it showed the same trend to NPP results. Fig. 2 illustrates average carbon absorption of the reservoir on the day of sample collection (with a 95% confidence interval). The highest CO_2 absorption (5,777±346 $mg.m^{-2}.day^{-1}$) was observed in the same day of the highest NPP on March 29, 2019, while the lowest value (350±175 $mg.m^{-2}.day^{-1}$) was recorded on January 11, 2019. It appears that eutrophication or phytoplankton blooms in aquatic ecosystems can decrease the amount of CO_2 emitted into the atmosphere, and in turn, increase carbon sequestration in waters (Pacheco et al., 2013; Weinke et al., 2014). However, once these phytoplankton decay, the process of decomposition may deplete the concentration of dissolved oxygen above lake beds and provoke the anaerobic activity of microbes that subsequently generates greenhouse gases such as methane (Borges et al., 2015).

In terms of relationships between CO_2 absorption in biomass and physicochemical variables, there were significant positive linear relationships between log-transformed CO_2 absorption and temperature ($r^2 = 0.514$, $p < 0.01$) as well as light intensity ($r^2 = 0.652$, $p < 0.01$) (Fig. 3 and 4). Light and temperature are known to be important abiotic drivers of algal growth and accordingly photosynthesis (Wetzel, 2001). On the other hand, neither transparency nor total phosphorus showed a significant relationship with CO_2 absorption ($p > 0.05$). This is attributed to an abundance of total phosphorus that could appear in the form of colloidal particles in the reservoir (Heathwaite et al., 2005). As a result, the increase of non-algal turbidity reduces water transparency without a corresponding relationship with NPP. A multiple regression equation using log-transformed variables was derived to describe the influence of temperature and light intensity on CO_2 absorption as follows:

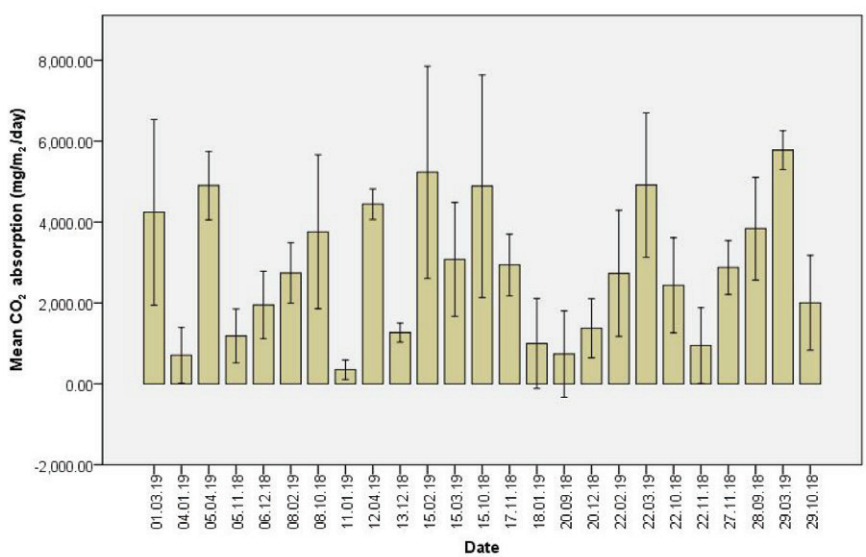

Fig. 2 Variations of CO_2 absorption (mg. m^{-2}. d^{-1}) during the period of study (Error bars represent the 95% confident interval (CI))

$$\ln(CO_2 abs) = -11.347 + 1.197*\ln(Light) + 3.965*\ln(Temp) \quad (5)$$

where ln = Natural logarithm;
CO_2 abs = CO_2 absorption (mg. m^{-2}. day^{-1});
Light = Average daily light intensity (W. m^{-2})
Temp = Water temperature (°C)

The coefficient of determination (r^2 = 0.734) was relatively high implying that the equation can explain 73.4% of the variation of CO_2 absorption as phytoplankton biomass. The relationship described by the equation was highly significant ($p < 0.01$).

TSI calculated from total phosphorus ranged between 69.0 and 85.4 while TSI from Secchi transparency ranged from 75.4 to 80.4. The results of TSI as individual surrogate indicators categorized the reservoir as eutrophic (50≤TSI≤70) to hypertrophic (TSI>70). The high index value of total phosphorus indicated that the Sakaew reservoir is enriched by the nutrient; and thus, the growth of phytoplankton in the reservoir is not limited by phosphorus. It is consistent with Tonetta et al. (2015) suggesting that there is no significant correlation between nutrients (nitrogen and phosphorus) and NPP in nutrient rich tropical lakes. By contrast, other factors, such as water temperature and light intensity, tend to play more important roles. In addition, the values of TSI where TSI (TP) is close to TSI (SD) indicate that non-algal particulates or dissolved color may dominate light attenuation (Carlson, 1992). The reason is that, in most turbid lakes, a close relationship between TSI (TP) and TSI (SD) results from clay particles that contain phosphorus while the phytoplankton are unable to utilized all the phosphorus and play a less significant role in contributing to the light attenuation (Carlson & Simpson, 1996). In other words, not all the measured phosphorus is utilized by the phytoplankton.

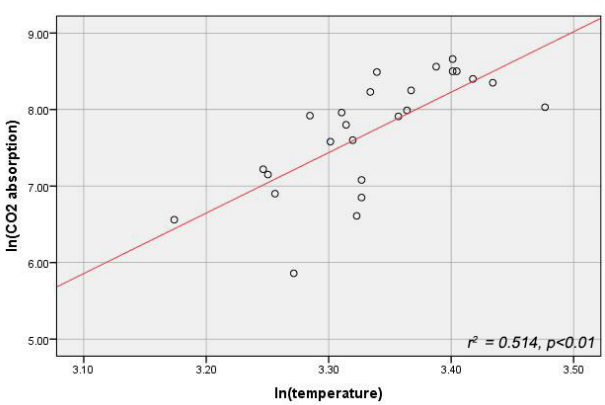

Fig. 3 Relationship between $\log_e$-transformed CO_2 absorption and water temperature

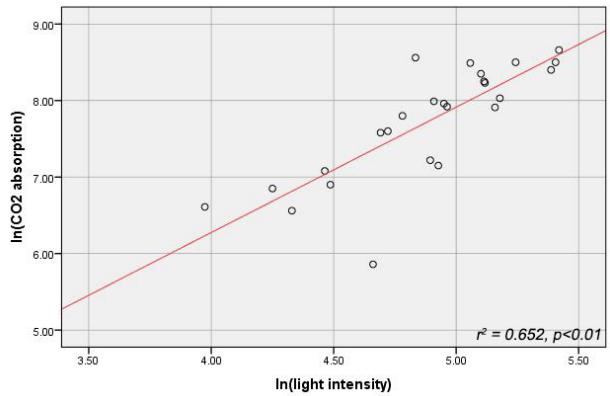

Fig. 4 Relationship between $\log_e$-transformed CO_2 absorption and light intensity

Conclusion

Even though freshwater ecosystems constitute a small fraction of the earth in terms of surface area, they play a crucial role in the global carbon cycle and can be regarded as a potential and promising solution for CO_2 capture and storage. Phytoplankton as the major producers in aquatic ecosystems fix CO_2 via photosynthesis to produce their biomass or NPP. Therefore, the measurement of primary productivity is essential to understand energy and nutrient flows as well as the carbon cycle in the systems. The findings from the present study reaffirmed the importance of aquatic ecosystems. The results indicated that CO_2 captured by phytoplankton in Srakaew reservoir ranged from 350 to 5,777 mg.m^{-2}.day^{-1} (mean = 2,813 mg.m^{-2}.day^{-1}). CO_2 absorption displayed a significant linear relationship with light intensity and water temperature. Likewise, seasonal variation can affect NPP and CO_2 absorption. In the hot season, NPP and CO_2 absorption showed a significantly higher value than the cool season while there was no significant difference in the rainy season compared the other seasons. According to the trophic state assessment, Srakaew reservoir was classified as eutrophic and hypereutrophic due to its low Secchi transparency coupled with high nutrient levels in the water. However, although NPP can roughly estimate CO_2 captured in an ecosystem in the form of producer biomass, it may not usually be a good index to identify the carbon sequestration in the whole system since heterotrophic respiration, namely carbon losses by herbivory and the decomposition of dead organic matter, is not yet considered. Therefore, additional studies associated with

heterotrophic respiration are required to fulfill the gap. As the present study was conducted under a limited period of time, temporal variation in production in a longer scale of time should be evaluated. Where possible, other parameters that were excluded from this study, for instance, chlorophyll a, nitrogen content and species composition of phytoplankton should be taken into consideration.

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Antioxidant Activity, Total Phenolic Content and Anti-Tyrosinase Activity of Thai Colored Rice Cultivar Extracts

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Abstract

This study aimed to evaluate the antioxidant activity, total phenolic content and tyrosinase inhibitory activity of Thai colored rice. Two different colored rice cultivars (Sew-Dang and Nor-Prae) were extracted using water and 95% ethanol. Antioxidant activities of rice extracts were tested using three different methods: DPPH, ABTS and FRAP. These analyses revealed that ethanolic extracts produced stronger antioxidant activity than water extracts. The ethanolic extract of Nor-Prae rice grains contained 114.12 mg GAE/g extract, 147.55 mg TE/g extract, and 152.44 mg $FeSO_4$/g extract tested by DPPH, ABTS and FRAP assays respectively which were significantly higher than Sew-Dang rice ($P<0.05$).Total phenolic content was determined using the Folin-Ciocalteu method, and the ethanolic extract had significantly greater levels of phenolics than water extracts ($P<0.05$). Nor-Prae ethanolic extracts were determined to possess the greatest phenolic content, which was 39.18 mg GAE/g extract, relative to other extracts assessed. Tyrosinase inhibitory activities of rice extracts were determined using the dopachrome microplate method. The greatest tyrosinase inhibitory activity was produced by the ethanolic extract of Nor-Prae rice, which inhibited tyrosinase 24.19%, while all water extracts had no affect on the activity of tyrosinase. In summary, the ethanolic extract of Nor-Prae rice had the strongest antioxidant capacity, greatest total phenolic content and the greatest capacity to inhibit tyrosinase activity. This indicated the potential of colored rice as a source of natural antioxidants and tyrosinase inhibitors, which might be used for further cosmetic or pharmaceutical product development.

Introduction

Rice (*Oryza sativa* L.) belongs to the Poaceae family, and is a staple food consumed in Asia and is also the most important export of Thailand. Generally, rice contains nutraceuticals such as vitamin E, vitamin B complex, minerals, fiber and important amino acid (Liu, 2007; Yodmanee et al., 2011). Colored rice is a good source of phytochemical components such as phenolic compounds, anthocyanins and γ- oryzanol, which has been reported to be an efficient antioxidant (Chakuton et al., 2012). Several studies have shown that colored

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rice exhibits greater antioxidant activity and contains more potent antioxidant compounds, including anthocyanins and phenolic compounds, than white rice (Ahuja et. al., 2007; Vichapong et al., 2010; Chakuton et al., 2012). Antioxidant compounds promote health by protecting the cells of the body from damage caused by free radicals and reactive oxygen species. Moreover, previous studies have shown that antioxidant activity correlates well with total phenolic content in rice (Yodmanee et al., 2011; Nakornriab, 2018). The most prominent phenolic compounds present in colored rice have been reported to be anthocyanins (Iqbal et al., 2005; Zhang et al., 2006; Yawadio et al., 2007). Therefore, it is important to determine the antioxidant activity and total levels of phenolic compounds in Thai colored rice cultivars.

Tyrosinase is a copper-containing enzyme involved in the production of melanin. This enzyme catalyzes the oxidation of L-tyrosine to 3-(3,4-dihydroxy-phenyl)-L-alanine (L-DOPA) and oxidation of L-DOPA to produce dopachrome, which is results eventually in the production of melanin pigment (Kim & Uyama, 2005). High levels of production of tyrosinase enzyme leads to a dermatological disorder such as age spots, melanoma, freckles and hyperpigmentation (Costin & Hearing, 2007; Ortonne & Bissett, 2008). Moreover, tyrosinase also is responsible for the enzymatic browning of fruits and vegetables, which results in discoloration. This is an unfavorable characteristic and results in economic and nutritional losses (Martinez & Whitaker, 1995). The identification of tyrosinase inhibitors may be important for the production of cosmetic products as well as the food industry (Wang et al., 2011; Loizzo et al, 2012). Anti-tyrosinase compounds are derived from both synthetic and natural sources such as kojic acid, hydroquinone, arbutin, ellagic acid, and ascorbic acid (Zolghadri et al., 2019). Moreover, several phenolic compounds have been reported to contain antioxidant activity along with anti-tyrosinase activity in various plant species (Rashed et al., 2016; Chatatikun & Chiabchalard, 2017). Although various Thai colored rice cultivars have been reported to have tyrosinase inhibitory activity (Jansom et al., 2010), some varieties are weak tyrosinase inhibitors (Teeranachaideekul et al, 2018). Further, there is no assessment of the bioactive activity and phytochemical components within Sew-Dang and Nor-Prae rice cultivars. Sew-Dang rice (*Oryza sativa* L. cv. Sew-Dang) and Nor-Prae rice (*Oryza sativa* L. cv. Nor-Prae) are glutinous rice cultivars cultivated in Chiang Rai, Thailand. Both rice cultivars have a deep red bran layer (Chaichana, 2019). This study aimed to evaluate antioxidant activity using various antioxidant models. Furthermore, the total phenolic content and tyrosinase enzyme inhibition of water and ethanolic extracts of Thai rice cultivars were also determined.

Materials and Methods

1. Sample preparation

Sew-Dang (*Oryza sativa* L. cv. Sew-Dang) and Nor Prae rice (*Oryza sativa* L. cv. Nor-Prae) were collected from Chiang San district, Chiang Rai, Thailand. The rice grains were dried at 60°C. Dried samples were ground into a fine powder (100 g) and extractions were performed using distilled water and 95% ethanol (1:10 w/v) at room temperature with frequent agitation for 24 h. The mixture was filtrated through Whatman no. 1 filters. Crude rice extracts were obtained from filtrates that were evaporated using a rotary evaporator under reduced pressure and lyophilized via freeze-drying.

2. Antioxidant activity

2.1 DPPH radical scavenging assay

The DPPH radical scavenging ability of rice extracts was determined according to the modified method of Brand–Williams et al. (1995) and Ho et al. (2010). Briefly, 0.5 mL of various concentrations of plant extracts in methanol were added to 1.5 mL of 0.1 mM DPPH in methanol. The mixtures were incubated in the dark at room temperature for 20 min. Absorbance was measured at 517 nm using UV/Visible spectrophotometry (Biochrom Libra S60, UK). The percentage of free radical inhibition provided by the extract was calculated using the following equation:

$$\% \text{ Inhibition} = [(A- (B-C)/A] \times 100 \quad (1)$$

Where A is the absorbance of the control (DPPH solution), B is the absorbance of the tested sample (the plant extract with DPPH solution), and C is the absorbance of the blank sample (the plant extract without DPPH solution).

The IC_{50} value was defined as the concentration of the sample required to scavenge 50% of DPPH radicals. The IC_{50} was obtained from the linear regression of the dose-response curve of % inhibition versus concentration. Then, the antioxidant activity of rice extracts was reported as the gallic acid equivalent

antioxidant capacity per gram extract (mg GAE/g extract), as follows:

$$\text{Antioxidant activity (mg GAE/g extract)} = \frac{(IC_{50}\ \text{gallic acid (mg/mL)})}{(IC_{50}\ \text{rice extract (mg/mL)})} \times 1000 \quad (2)$$

2.2 ABTS radical scavenging assay

The ABTS radical scavenging activity was measured by assessing the color change associated with the formation of an ABTS cation radical ($ABTS^{\bullet+}$), with slight modifications (Re et al., 1999). The $ABTS^{\bullet+}$ was generated via a reaction between 7 mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was incubated in the dark at room temperature for 12-16 h before used. Afterward, the ABTS working solution was diluted with 95% ethanol until the solution produced an absorbance of 0.700 ± 0.02 at 734 nm. To assess experimental replicates, 20 µL of various concentrations of rice extracts were mixed with the $ABTS^{\bullet+}$ working solution and incubated in the dark for 6 min before their absorbance was measured at 734 nm using UV/Visible spectrophotometry (Biochrom Libra S60, UK). Trolox solution was used as a standard. The percentage of free radical inhibition of rice extracts were calculated using the following equation:

$$\%\ \text{Inhibition} = [(A-(B-C)/A] \times 100 \quad (3)$$

Where A is the absorbance of the control (ABTS solution), B is the absorbance of the tested sample (the extracts with ABTS solution), and C is the absorbance of the blank sample (the extract without ABTS solution).

The IC_{50} value was defined as the concentration of the sample required to scavenge 50% of ABTS radicals. The IC_{50} was obtained from the linear regression of the dose-response curve of % inhibition versus concentration. The antioxidant activity of rice extracts was reported as the trolox equivalent antioxidant capacity per gram extract (mg TE/g extract).

$$\text{Antioxidant activity (mg TE/g extract)} = \frac{(IC_{50}\ \text{trolox (mg/mL)})}{(IC_{50}\ \text{rice extract (mg/mL)})} \times 1000 \quad (4)$$

2.3 Ferric reducing antioxidant power (FRAP) assay

Reducing power was determined using a ferric reducing antioxidant power (FRAP) assay described by Benzie & Strain (1996), with some modifications. Briefly, extracts were dissolved in 95% ethanol and 1.0 mg/mL concentrations of extracts were obtained. Then, an aliquot of 500 µL of rice extract was mixed with 1.5 mL FRAP reagent (10 mM TPTZ solution, 20 mM $FeCl_3.6H_2O$, 300 mM acetate buffer, pH 3.6 and deionized water). Next, mixtures were incubated in the dark 15 min and measured at 593 nm using a UV/Visible spectrophotometer (Biochrom Libra S60, UK). Deionized water was used as a blank solution. Reducing power was calculated from a $FeSO_4$ standard curve and expressed as mg $FeSO_4$/g extract.

3. Total phenolic compound content assay

Total phenolic content was determined using the Folin-Ciocalteu method, with some modifications (Chandler & Dodds, 1983). In brief, 0.25 mL rice extract (1 mg/mL) was mixed with 1.25 mL water, 0.25 mL 95% ethanol and 0.125 mL 50% Folin-Ciocalteu reagent. Mixtures were incubated 5 min at room temperature, 0.25 mL 5% Na_2CO_3 was added and mixtures were incubated in the dark for 1 h. The absorbance of each solution was measured at 725 nm using 95% ethanol as a blank. Total phenolic content was calculated from a gallic acid standard curve and expressed as mg gallic acid equivalents (mg GAE/g extract).

4. Tyrosinase inhibitory assay

Tyrosinase inhibitory activity of rice extracts was evaluated using the dopachrome microplate method (Potduang et al., 2008). Rice extracts were diluted with 20% ethanol to a final concentration of 1 mg/mL. Then, 50 µL of each rice extract was mixed with 150 µL 20 mM phosphate buffer (pH 6.8) and 50 µL mushroom tyrosinase (313 units/mL). Mixtures were incubated at 37°C for 10 min. Afterward, 50 µL of 0.34 mM 3,4-Dihydroxy-L-phehylalanine (L-DOPA) was added to each well and incubated at 37°C for an additional 10 min. Absorbance was measured at 492 nm using a microplate reader (M965+, Metertech, Taiwan). Kojic acid (1 mg/mL) was used as a positive control. Percentage tyrosinase inhibition was calculated using the following equation:

$$\text{Tyrosinase inhibition (\%)} = [(A-B)-(C-D)/A-B] \times 100 \quad (5)$$

Where A is the absorbance of the control (L-DOPA, tyrosinase); B is the absorbance of the blank (L-DOPA); C is the absorbance of the sample (rice extract, L-DOPA and tyrosinase) and D is the blank for C (L-DOPA mixed with rice extract).

5. Statistical analysis

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

Results and Discussion

1. Antioxidant activity

In this study, two glutinous colored rice cultivars (Sew-Dang and Nor-Prae) were extracted using water and 95% ethanol. The water and ethanolic extracts of both rice cultivars were tested for their antioxidant activity, total phenolic content, and tyrosinase inhibitory activity. Various methods have been used to analyze the antioxidant capacity in several plant materials, and since they all function based on different reaction mechanisms results of each type of test may differ (Pérez-Jiménez & Saura-Calixto, 2006). Therefore, at least two test methods should be used to produce results that reliably indicate the antioxidant activity of plant samples (Pérez-Jiménez et al., 2008). In this study, three different methods including DPPH, ABTS and FRAP were used to analyze the antioxidant capacity of rice extracts.

The DPPH method has been widely used to provide standard information regarding the antioxidant activity of various plant species. DPPH is a stable nitrogen radical species that is capable of accepting either electron or hydrogen radicals to form a stable diamagnetic molecule. Antioxidants are able to reduce stable radical DPPH to is yellow-colored, non-radical form, DPPH-H through their hydrogen donating capabilities (Cotelle et al., 1996). The results of DPPH radical scavenging activity indicated the concentration of the sample required to scavenge 50% of DPPH radicals (IC_{50}) and was expressed as the gallic acid equivalent antioxidant capacity per gram extract (mg GAE/g extract). The lower the IC_{50} value, the greater antioxidant activity it represented. As shown in Table 1, gallic acid which was used as a standard compound had the lowest IC_{50} value of 0.006 mg/mL. Among colored rice extracts, the ethanolic extract of Nor-Prae rice had the lowest IC_{50} (0.05 mg/mL), followed by the ethanolic extract of Sew-Dang rice (0.11 mg/mL) and the water extract of Nor-Prae rice (0.75 mg/mL). The highest IC_{50} was found in the water extract of Sew-Dang rice (1.39 mg/mL). For antioxidant activity, our results demonstrated that the significantly ($P<0.05$) greatest antioxidant activity (114.12 mg GAE/g extracts) was attributed to the ethanolic extract of Nor-Prae rice, followed by the ethanolic extract of Sew-Dang rice (53.66 mg GAE/g extracts). Water extracts of both types rice were determined to contain radical scavenging activities of 7.63 and 4.11 mg GAE/g extract, respectively, which was significantly lower than activities of ethanolic extracts ($P<0.05$).

Table 1 DPPH radical scavenging activity of water and ethanolic extracts of Sew-Dang and Nor-Prae rice cultivars

Rice cultivars	Extracts	IC_{50} (mg/mL)	DPPH radical scavenging activity (mg GAE/g extract)
Sew-Dang	Water	1.39 ± 0.067^{a}	4.11 ± 0.206^{c}
	Ethanol	0.11 ± 0.004^{c}	53.66 ± 1.996^{b}
Nor-Prae	Water	0.75 ± 0.011^{b}	7.63 ± 0.117^{c}
	Ethanol	0.05 ± 0.022^{d}	114.12 ± 4.569^{a}
Gallic acid	-	0.006 ± 0.000^{d}	-

Remark: The data are given as mean ± standard deviation (SD) of triplicate data. Values followed by different letters in column were significantly different ($P<0.05$).

ABTS is another method that has been widely used to measure the radical scavenging activity of antioxidant compounds. ABTS can be oxidized by potassium persulfate or manganese dioxide, which gives rise to the ABTS cation radical ($ABTS^{\bullet+}$). The ABTS method measures the ability of the sample to donate an electron or hydrogen to $ABTS^{\bullet+}$ (blue-green color) to form ABTS (colorless) (Moon & Shibamoto, 2009; Alam et al., 2013). The $ABTS^{\bullet+}$ radical scavenging activity in term of the extract concentration required to inhibit 50% of initial ABTS radical (IC_{50}) and was expressed as mg trolox equivalent antioxidant activity per gram of extract (mg TE/g extract). As shown in Table 2, ABTS assays revealed that the radical scavenging activities of rice extracts were similar to those of the DPPH assay. Trolox was used as a standard antioxidant compound and gave the lowest IC_{50} with 0.35 mg/mL ($P<0.05$). Furthermore, the result showed that the IC_{50} of colored rice extracts ranged from 2.39-97.75 mg/mL. The lowest IC_{50} among colored rice extracts was found in the ethanolic extract of Nor-Prae rice with IC_{50} value of 2.39 mg/mL followed by the ethanolic extract of Sew-Dang rice with IC_{50} of 5.94 mg/mL. However, both water extracts had higher IC_{50} values with 54.57 mg/mL (Nor-Prae) and 97.75 mg/mL (Sew-Dang). The ABTS radical scavenging activity of the ethanolic extract of Nor-Prae rice was 147.55 mg TE/g extract, which was significantly stronger than the ethanolic extract of Sew-Dang rice, which was 59.23 mg TE/g extract ($P<0.05$). The aqueous extracts of rice types, Nor-Prae and Sew-Dang, exhibited weak antioxidant activity, which was determined to be 6.45 and 3.60 mg TE/g extract, respectively.

Table 2 ABTS radical scavenging activity of water and ethanolic extract of Sew-Dang and Nor-Prae rice cultivars

Rice cultivars	Extracts	IC_{50} (mg/mL)	ABTS radical scavenging activity (mg TE/g extract)
Sew-Dang	Water	97.75 ± 1.329[a]	3.60 ± 0.049[c]
	Ethanol	5.94 ± 0.099[c]	59.23 ± 0.998[b]
Nor-Prae	Water	54.57 ± 1.142[b]	6.45 ± 0.134[c]
	Ethanol	2.39 ± 0.089[d]	147.55 ± 5.543[a]
Trolox	-	0.35 ± 0.002[e]	-

Remark: The data are given as mean ± standard deviation (SD) of triplicate data. Values followed by different letters in column were significantly different (P<0.05).

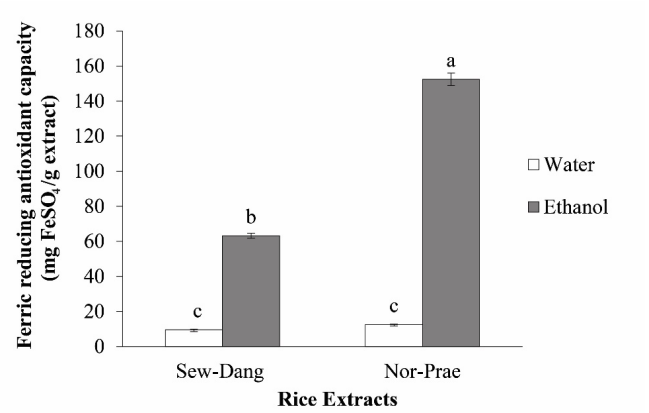

Fig. 1 Ferric reducing antioxidant power of water and ethanolic extracts of Sew-Dang and Nor-Prae rice cultivars. Each bar represents the mean of three replicates and the error bars indicate the standard error of the means. Values labelled with different letters were significantly different (P < 0.05).

Also, a FRAP assay was used to measure the quantity of antioxidants or reductants present within rice extracts. Antioxidants can reduce ferric (III) ions to ferrous (II) ions via a redox-linked colorimetric reaction (Benzie & Strain, 1996; Li et al., 2006). The ferric reducing antioxidant power of colored rice is provided Fig. 1. The ethanolic extracts of Nor-Prae rice had the greatest (P<*0.05*) ferric reducing capacity (152.44 mg $FeSO_4$/g extract), followed by that of the ethanolic extract of Sew-Deng rice, which was determined to be 63.24 mg $FeSO_4$/g extract. Again, water extracts of Nor-Prae and Sew-Dang rice had low levels of ferric reducing capacity, and values determined for the extracts were 12.64 and 9.61 mg $FeSO_4$/g extract, respectively. The antioxidant activities of Sew-Dang and Nor-Prae rice cultivars have not been reported. However, other colored rice cultivars demonstrated good sources of antioxidant compounds. According to the previous report, Vichit and Saewan (2015) revealed that some black and red rice cultivars gave strong antioxidant activity ranging from 0.06 - 1.36 mg AAE/mL for FRAP, IC_{50} 0.10 - 1.12 mg/mL for DPPH and 7.57- 40.48 % for TBARS. A similar result was reported by Moko et al. (2014) that the colored rice had higher antioxidant activity than non-colored varieties. It was shown that the red rice varieties had the highest DPPH radical scavenging ability with 88.29% and also had the lowest IC_{50} with 26.26 µg/mL.

2. Total phenolic compound content assay

Phenolic compounds are commonly found in plants and have been reported to have several biological functions including antibacterial and antioxidant activities (Soobratte et al., 2005). The main phenolics in colored rice cultivars are phenolic acids such as ferulic, coumaric, caffeic, cinnamic and gallic acids (Tian et al., 2005; Zhou et al., 2004). Also, another phenolic in colored rice is mainly anthocyanin, which has strong antioxidant activity (Goufo & Trindade, 2014). Phenolic compounds act as antioxidants, since they are able to scavenge free radicals, to donate hydrogen atoms or electrons and to chelate metal cations (Javamardi et al., 2003). Previous publications have shown that antioxidant activity correlates with phenolic content in several plant species (Velioglu et al., 1998; Zhang et al., 2006; Yawadio et al., 2007; Do et al., 2014). As shown in Fig. 2, investigations of the phenolic content of rice extracts produced similar results as the DPPH, ABTS and FRAP assays. This suggests that the antioxidant activity of colored rice likely depends on total phenolic content. The highest levels of phenols were observed in the ethanolic extract of Nor-Prae rice, followed by the ethanolic extract of Sew-Dang rice in which levels were determined to be 39.18 and 22.41 mg GAE/g extract, respectively. Additionally, water extracts of the both types of rice contained significantly (P<*0.05*) lower levels of phenolics: 4.43 and 2.32 mg GAE/g extract, respectively. These findings are consistent with a previous report by Yodmanee et al. (2011), which showed that the antioxidant capacity of dehusked rice grain extracts were correlated with polyphenol content. Furthermore, Nakornriab (2018) reported that brown rice extracts had the highest total phenolic content and antioxidant activity. The researchers further showed that total phenol content and antioxidant activity was tightly correlated.

Solvents used to extract bioactive compounds of plants have an effect on the resulting biological activities of

extracts. Most phenolic substances range from polar to nonpolar in plants, thus the choice of solvent for extractions is very important for phenolic compounds (Do et al., 2014). A previous study reported that the majority of solvents used for extracting antioxidant compounds are comprised of mixtures of organic solvents including ethanol, methanol, and acetone. Ethanol has previously been shown to be a good solvent for extracting antioxidant compounds from plant materials. The studies also suggested that ethanol is the most suitable nontoxic solvent for extracting the compounds (Dai & Mumper, 2010). In this study, all ethanolic extracts obtained had higher antioxidant capacities than water extracts when any of the three measures of antioxidant capacity were considered. Also, ethanolic extracts had greater phenolic content than water extracts. This is consistent with a previous report, which showed that ethanolic extracts of colored rice (Sang-Yod red rice) produced strong DPPH radical scavenging activity and also had high total phenolic and flavonoid content (Hansakul et al., 2011). This finding suggests solvent used for extraction plays an important role in determining antioxidant activity and phenolic content, which is due to differences in the solubilities of compounds within samples. However, the antioxidant activity and concentration of polyphenol compounds also depend on the cultivars of colored rice used. In the present study, Nor-Prae rice extracts possessed significantly ($P<0.05$) greater antioxidant capacities and had significantly greater levels of total phenolic content than Sew-Dang rice extracts.

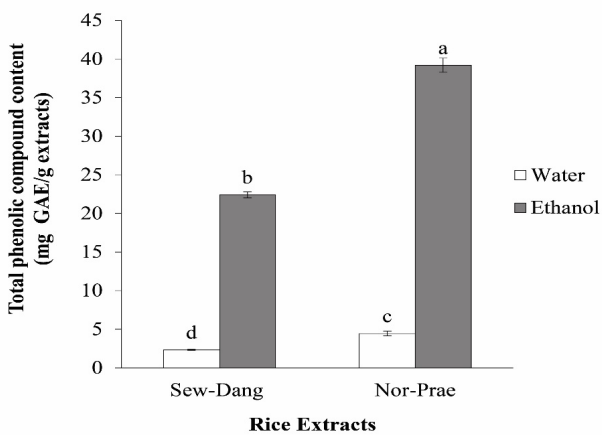

Fig. 2 Total phenolic compound content of water and ethanolic extracts of Sew-Dang and Nor-Prae rice cultivars. Each bar represents the mean of three replicates and error bars indicate the standard error. Values labelled with different letters were significantly different ($P < 0.05$).

3. Tyrosinase inhibitory assay

Tyrosinase is a polyphenol oxidase enzyme that is involved in melanin biosynthesis in organisms. Overproduction of tyrosinase leads to hyperpigmentation in human and animal skin and also affects the fruit and vegetable quality (Chang, 2009). Tyrosinase is a copper-containing enzyme that catalyzes the conversion of L-tyrosine to L-DOPA, and the oxidation of L-DOPA to dopaquinone, which are required for melanin biosynthesis (Kim & Uyama, 2005). Therefore, identification of a tyrosinase inhibitor has the potential to inhibit the process of hyperpigmentation. In our study, the ability of rice extracts to inhibit tyrosinase activity was evaluated using a dopachrome assay, and L-DOPA was used as a substrate of the tyrosinase enzyme. The results of the tyrosinase inhibitory activity assay are shown in Table 3. The tyrosinase inhibitory activity of rice extracts was influenced by rice cultivar and solvents used for extractions. Anti-tyrosinase activity of rice extracts were compared to 1 mg/mL kojic acid (positive control). At this concentration, both rice extracts had a lower inhibitory activities against tyrosinase than kojic acid, which had the significantly ($P<0.05$) highest tyrosinase inhibitory activity assessed, with percentage inhibition of 92.74%. Among rice extracts tested, the ethanolic extracts of Nor-Prae and Sew-Dang rice displayed significant anti-tyrosinase activity while water extracts of both rice cultivars did not affect tyrosinase activity because their activities were so low. The highest level of tyrosinase inhibitory activity was determined to be associated with the ethanolic extract of Nor-Prae rice, which had a percentage inhibition of 24.19%. The ethanolic extract of Sew-Dang rice exhibited a low inhibitory activity against the tyrosinase enzyme, with a percentage inhibition value of 6.45%. This result is consistent with reports of Jansom et al. (2010), who showed that some purple glutinous rice extracts showed strong tyrosinase inhibitory activity. Similar anti-tyrosinase abilities were also reported in red rice (*Oryza nivara*) extracts (Batubara et al., 2017). Several polyphenol compounds found in natural sources have been shown to be effective tyrosinase inhibitors including gallic acid, kaempferol, quercetin, catechin, and rhamnetin (Orhan & Khan, 2014; Lee et al., 2016; Panzella & Napolitano, 2019). Miyazawa et al. (2003) demonstrated that protocatechuic acid methyl ester isolated from black rice bran had strong tyrosinase inhibitory activity.

Table 3 Tyrosinase inhibitory activity of water and ethanolic extracts of Sew-Dang and Nor-Prae rice cultivars

Rice cultivars	Extracts	Tyrosinase Inhibition (%)
Sew-Dang	Water	ND
	Ethanol	6.45 ± 1.40^{c}
Nor-Prae	Water	ND
	Ethanol	24.19 ± 1.40^{b}
Kojic acid	-	92.74 ± 2.42^{a}

Remark: The data are given as mean ± standard deviation (SD) of triplicate data. Values followed by different letters were significantly different ($P<0.05$), ND: Not determined at assayed concentration (consequence of low activity).

Conclusion

The present study indicates that the biological activities of colored rice extracts depend on the type of rice cultivar used and the solvent used for extractions. The ethanolic extracts had more potent antioxidant activity, greater total phenolic content and increased tyrosinase inhibitory activity relative to water extracts. The ethanolic extract of Nor-Prae rice produced the strongest antioxidant activity according to all three methods used to measure antioxidant capacity. Further, it also had the highest total phenolic content and the greatest tyrosinase inhibitory activity of all extracts examined. Therefore, this research suggests that Nor-Prae rice extracts contain potent of antioxidant compounds and tyrosinase inhibitors, which are likely phenolics. The HPLC quantitative analysis and stability test of colored rice extract should be investigated in further works in order to achieve pharmaceutical product development.

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Effects of Nevadensin from Rice Paddy Herb *(Limnophila aromatica)* on Growth and Physiological Parameters of Hybrid Catfish *(Clarias macrocephalus × C. gariepinus)*

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Abstract

This study examined the effects of dietary supplementation of nevadensin, a flavonoid isolated from rice paddy herb (*Limnophila aromatica*), on growth and physiological parameters of hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*). Fish (initial weight of 5.00 ± 1.00 g) were fed with the diets containing 0 (control), 10, 30, and 50 mg nevadensin/kg feed for 8 weeks. Growth parameters, feed intake, and feed efficiency were significantly improved (P<0.05) by the diets containing 30 and 50 mg nevadensin. Survival rate and organosomatic indices for the intestine, gill, heart, kidney, and spleen were not significantly different (P>0.05) among the groups. However, the hepatosomatic index in fish fed with 30 mg nevadensin/kg diet was significantly increased (P<0.05) when compared with the control. A significant improvement (P<0.05) in villi heights, muscular thicknesses, goblet cells, and microvilli heights was detected in fish fed with nevadensin containing diets. Villi widths, total enterocyte heights and supranucleus heights decreased significantly (P<0.05) in fish fed with nevadensin supplemented diets. Red blood cells and white blood cells in fish fed with supplemented diets were significantly higher (P<0.05) than those in fish fed with the control diet. Hemoglobin and hematocrit significantly increased (P<0.05) in fish fed with 50 mg nevadensin/kg diet compared to the control. The highest level of lymphocyte was observed in fish fed with 10 mg nevadensin. Blood biochemical values were not significantly different (P>0.05) in any of the treated groups compared to the control. In conclusion, the results indicate that nevadensin could be used as a natural feed additive to improve somatic growth and physiological parameters of hybrid catfish. By using the second-order polynomial regression analysis, the optimal concentration of nevadensin for cultured catfish was estimated to be between 30.64 and 31.14 mg/kg diet.

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Introduction

Aquaculture products are recognized as a high-quality source of several essential nutrients for human consumption such as protein, omega-3, selenium, and vitamin D (Gupta, 2006). As an increase in worldwide demand for aquatic animals, intensified cultures have been developed to increase the production of fish (Van Hai, 2015). However, under intensive rearing conditions, farmed fish may expose to different stress conditions including water qualities, pathogens, and infectious diseases which have been reported to impair normal physiological functions (Pês et al., 2016). The use of antibiotics and synthetic compounds in aquaculture to improve fish growth and health is widely concerned due to their residues in fish products and surrounding environments (Gupta, 2006; Li et al., 2019). Additionally, inappropriate uses of drugs in fish cultivation could increase the relevance of drug-resistant organisms which may produce detrimental effects on both humans and animals (Abdel-Tawwab et al., 2018; Reverter et al., 2014; Zhou et al., 2015).

Natural additives obtained from plant-based products could be used to replace synthetic drugs or chemical compounds in aquaculture industries because of their lower side effects and toxicity (Adel et al., 2015; Amer et al., 2018; Hoseini et al., 2018; Wongtha et al., 2019). Herbal plants have long been used in folk medicine for a wide variety of many therapeutic proposes (Munglue, 2015; Munglue et al., 2019a). Several classes of bioactive compounds such as alkaloids, flavonoids, polyphenols, essential oils, terpenoids, and steroids were isolated and tested for their biological activities (Aanyu et al., 2018; Hoseinifar et al., 2020a; Jahazi et al., 2020; Villasante et al., 2016). In aquaculture, flavonoids from *Allium mongolicum,* curcumin, genistein, limonene, quercetin, cineole, thymol and rutin have positive effects on growth, nutrient assimilation and immunity of farmed fish (Aanyu et al., 2018; Amer et al., 2018; Hoseini et al., 2018; Jia et al., 2019; Li et al., 2019; Pês et al., 2015; Pês et al., 2016; Sruthi et al., 2018). However, the success of this approach depends largely on the correct doses, plant material forms, route of administration, optimal time of supplementation, life stages of fish and species-specific (Hoseinifar et al., 2020b; Van Hai, 2015; Zhou et al., 2015). Therefore, further investigations in this field are required.

Nevadensin (2-(4-methoxyphenyl)-5,7-dihydroxy-6,8-dimethoxy-4H-1-benzopyran-4-one) is a natural flavonoid that has been identified and isolated from many plant species including *Limnophila* species (Bui et al., 2004; Kukongviriyapan et al., 2007). It is considered as an effective substance that has been tested for its pharmacological potential and starting molecule in several drug development processes (Brahmachari & Gorai, 2006). It was indicated that nevadensin had an anti-hypotensive property in dog and cat models due to central and peripheral modulations (Song et al., 1985). Also, anti-inflammatory, anti-tumor, anti-cancer and anti-microbial properties of nevadensin have been identified by multiple scientific reports (Brahmachari et al., 2011; Chung & Geahlen, 1992; Yang et al., 1985; Reddy et al., 1991).

Hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) is an important freshwater fish that has been raised in several parts of Thailand because of its high growth rate and high market demand (Senanan et al., 2004). In 2017, the total production of catfish was around 105,144 tones and valued 5,126.6 million baht (Fishery Statistics Analysis and Research Group, 2019). Recently, catfish cultivation is now faced with uncontrollable disease outbreaks, various environmental changes and low water quality (Gabriel et al., 2019).

As the scientific data described above, nevadensin shows a variety of biological activities and more research on its pharmacological uses in both human and veterinary medicines are required. In aquaculture production sections, novel molecules that can be used as natural alternatives to synthetic compounds in the diets to improve growth performance, health, and general well-being of fish are also needed (Amer et al., 2018; Villasante et al., 2016). Thus, this research evaluated the effects of dietary nevadensin, a flavonoid isolated from rice paddy herb, on growth, feed efficiency, intestinal histology, hematology and serum biochemistry in hybrid catfish.

Materials and methods

1. Plant preparation, extraction, and isolation

Arial parts of rice paddy herb were harvested from Warinchumrap Distract, Ubon Ratchathani, Thailand, from August to September. A voucher specimen (Munglue 002) was kept at the Program of Biology, Faculty of Science, Ubon Ratchathani Rajabhat University, Thailand, for future reference. The plant samples were dried in a hot air oven at 60°C for 72 h and grounded into small pieces. Dried materials (1.8 kg) were macerated with

ethyl acetate for 72 h, filtrated with Whatman paper No.1, and the solvents evaporated using a rotary evaporator (Buchi, R-200, Switzerland) under reduced pressure, and dried using lyophilizer (Labconco Corporation, Missouri, USA). The crude extract (40.10 g) was chromatographed over silica gel (200 g). The column was eluted with *n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol, with a gradually increasing level of the more polar solvents, to give 17 groups of eluting fractions. The 6th subfraction (3.19 g) was then chromatographed over silica gel, eluted with *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol, with a gradually increasing quantity of the more polar solvents, to give 6 subfractions. Subsequently, the 5th subfraction was subjected to column chromatography on silica gel and eluted with *n*-hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol to afford 5 subfractions. The 3rd subfraction was further chromatographed to furnish nevadensin (0.0158 g) (Farkas et al., 1966; Suksamrarn et al., 2003). The characterization of nevadensin was performed by using ^{1}H and ^{13}C-Nuclear Magnetic Resonance (NMR). The data obtained from ^{1}H and ^{13}C-NMR by using dimethylsulfoxide as a solvent were ^{13}C: 182.2, 163.0, 162.3, 151.1, 148.3, 145.4, 131.6, 128.1, 128.0, 122.9, 114.7, 103.0, 102.8, 61.1, 60.0, and 55.5; ^{1}H NMR: 12.75 (s), 8.02 (d, J = 8.9 Hz), 7.14 (d, J = 8.9 Hz), 6.87 (s), 3.85 (s) and 3.76 (s). The chemical structure of nevadensin is presented in Fig. 1.

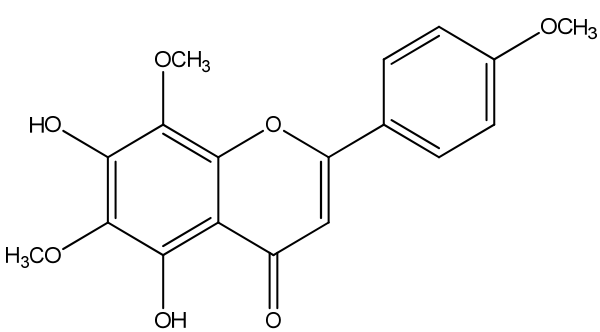

Fig. 1 Structure of nevadensin isolated from *L. aromatica*

2. Diet preparations

Fish diets containing 30% crude protein and 4% crude lipid were purchased from a local fish feed company (Centaco Group of Companies, Thailand). It is suggested that this protein level is recommended for the cultivation of catfish (Robinson et al., 2001). The range of nevadensin (0 (control), 10, 30, and 50 mg/kg diet) levels was determined according to the reports of Li et al. (2019) and Zhou et al. (2015). Each dose of nevadensin was mixed with the diets using cassava starch as a binder, moistened, minced, and oven-dried at 55°C for 24 h. The diet samples were kept in plastic bags at 4°C until use. The proximate composition of the experimental diets was analyzed according to the standard methods (Association of Official Agricultural Chemists [AOAC], 2012) and the results are shown in Table 1.

Table 1 Proximate analysis of the experimental diets

Nutritional composition expressed in dry weight basis (%)	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
Moisture content	6.96	6.55	5.85	7.87
Ash	8.86	9.16	9.13	8.72
Crude protein	31.61	32.80	31.29	30.91
Crude lipid	5.21	5.26	5.06	5.13
Crude fiber	1.56	1.28	1.38	1.15
Nitrogen-free extract	45.80	44.95	47.29	46.22

3. Fish preparations

Animal procedures were performed according to the guidelines provided by the National Research Council of Thailand. Hybrid catfish were obtained from Ubon Ratchathani Fishery Cooperative, Ubon Ratchathani, Thailand and acclimatized under laboratory conditions for 2 weeks. The fish (initial weight 5.00 ± 1.00 g) were randomly divided into four treatments of three replications and cultivated in the circle cement tanks (90 cm in diameter and 50 cm in height) with 255 L of water (20 fish/tank). Fish were fed ad libitum twice a day at 08.00 and 16.00 h for 8 weeks. Water qualities were maintained in the optimal range for catfish farming (dissolved oxygen 7.00 ± 0.05 mg/L, temperature, 29.00 ± 2.00°C, and pH, 7.20 ± 0.50) and checked daily by using ExStik® EC500 (Extech Instrument Corporation, U.S.A.). Three-quarters of water in each tank was siphoned daily to remove fish waste and replaced by clean water from a storage tank. Dead fish were removed and noted.

4. Growth parameters and survival

After 8 weeks of the feeding period, four fish from each tank were collected and weighed. Growth parameters including weight gain (WG), specific growth rate (SGR), average daily gain (ADG), feed intake (FI), feed conversion ratio (FCR) and survival rate (SR) were quantified using the following equations (Gabriel et al., 2019):

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

SGR (%/d) = 100 × [*In* final wet weight (g) – *In* initial wet weight (g)]/experimental days

ADG (g/d) = [final wet weight (g) – initial wet weight (g)] /experimental days

FI (g/fish) = feed intake (g)/number of fish

FCR = feed intake (g)/ weight gain (g)

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

5. Organosomatic indices

In this present study, clove oil was used as an anesthetic for catfish. Due to clove oil is insoluble in water, it was firstly dissolved in ethanol at a ratio of 1:9 (clove oil:95% ethanol) to create a 100 mg/ml solution. This stock solution was then mixed with dechlorinated water in an anesthetic bath to obtain the final concentration of 100 mg/L (Fawole et al., 2020).

After 24 h of the fasting period, four fish from each replication were individually anesthetized in the anesthetic bath supplied with continuous aeration for 10 min and monitored for behavioral responses as previously described by Hamácková et al. (2006). After exposure, the abdominal wall of anesthetized fish was opened. The liver, intestine, gill, heart, kidney, and spleen samples of hybrid catfish were carefully obtained, cleared from adipose tissues, and weighed. Then, organosomatic indices including hepatosomatic index (HSI), intestinosomatic index (ISI), gill somatic index (GSI), renosomatic index (RSI), and spleen somatic index (SSI) were determined based on the following formula (Wongtha et al., 2019):

Organosomatic indices (%) = 100 × [organ weight (g)/fish wet weight (g)]

6. Blood collection and preparation

At the end of the experiment, fish fasted for 24 h before blood collection. Four fish from each replicate were individually anesthetized in the anesthetic bath with aeration for 10 min. The blood sample was collected from the caudal vessel of anesthetized fish using a sterile syringe and divided into 2 parts. One part of the blood was transferred to 3 ml tubes containing 10% EDTA (ethylenediaminetetraacetic acid) as an anticoagulant for hematological evaluation. Another part of the blood was allowed to clot for 3 h at room temperature in a 1.5 ml microcentrifuge tube and was centrifuged at 3000 rpm at 4°C for 10 min. The serum was collected and stored at -20°C for further analysis.

7. Hematology

Hematological studies were performed as the methods suggested by Campbell & Ellis (2007). Red blood cells (RBC), white blood cells (WBC), and platelets were counted by using a Neubauer hemocytometer. Blood smears were prepared and stained with Wright- Giemsa. Differential white blood cell counts were evaluated under a light microscope. Hematocrit (HCT) was determined by using the microhematocrit method. The hemoglobin concentration (HGB) was determined by using the cyanmethemoglobin method. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were also calculated (Gabriel et al., 2015).

8. Serum biochemistry

The serum glucose level was determined by using Trinder's method (Barham & Trinder, 1972). Total protein content was measured by using the Biuret method (Gornall et al., 1949). Serum albumin was detected by using the Bromo Cresol Green (BCG) method (Leonard et al., 1971). Alkaline phosphatase (ALP) was analyzed using the method described by Tietz et al. (1983). Aspartate transaminase (AST) was evaluated according to Schumann et al. (2002). Triglyceride was estimated as reported by Cole et al. (1997). Creatinine content was tested using the Jaffe reaction (Bowers & Wong, 1980). Globulin content was calculated by subtracting the level of albumin from total protein (Valladão et al., 2017).

9. Intestinal histology

The intestinal samples were obtained from four fish of each replicate. They were cut at the end of the pyloric part of the stomach to 2 cm before the anus and subsequently divided into the proximal, middle, and distal parts. They were cleared from adjacent tissues, cleaned by using 0.90% normal saline, and preserved in 10% neutral buffered formalin. The specimens were subsequently dehydrated, embedded in the paraffin wax, cut into slices with a thickness of 5 µm, and mounted on the slides. The tissues were then stained by Hematoxylin and Eosin (H & E).

To evaluate the effects of nevadensin-enriched diets on intestinal macromorphology of hybrid catfish (Fig. 2A), villi height, villi width, total muscular thickness, inner circulatory smooth muscle, and outer longitudinal smooth muscle were observed by a light microscope and recorded on a computer using Dino Capture 2.0 software (Munglue et al., 2019a; Wongtha et al., 2019). Additionally, to study the effects on intestinal micromor-

phology (Fig. 2B), enterocyte height, supranucleus height, subnucleus height, nucleus height, nucleus width, goblet cell number, and microvilli height were measured as the method recommended by Escaffre et al. (2007).

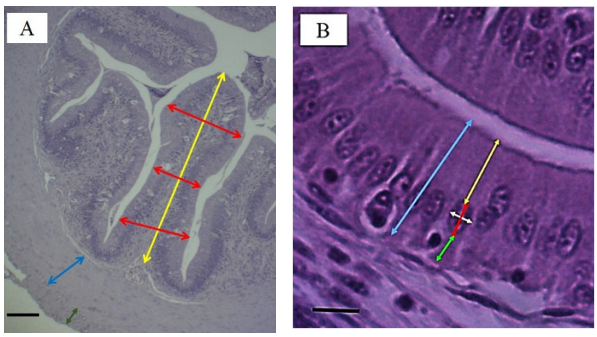

Fig. 2 Methodology for morphometric assessment of various parts of the intestine of hybrid catfish. (A) Intestinal macromorphology. Villi height (yellow line) was measured from the tip of the villi to the base, Villi width was measured from side to side of the villi at the top, the middle and the base and these values were then averaged to obtain a mean value of villus width, The thicknesses of the muscular layers including an inner circulatory layer (blue line) and an outer longitudinal smooth muscle (green line) were also evaluated. Scale bar = 200 µm. (B) Intestinal micromorphology. Measurement of enterocyte height (blue line), supranucleus height (yellow line), subnucleus height (green line), nucleus height (red line), and nucleus width (white line). Scale bar = 20 µm

10. Statistical analysis

Completely Randomized Design (CRD) was used in this research. The normal distribution of the data was evaluated using the Kolmogorov-Sminov test. To test the homogeneity of variances among the treatments, Levene's test was used (Pês et al., 2016). If the data were not normally distributed, the arcsin square-root transformation was established (Aanyu et al., 2018). Then, the data were analyzed by using one-way analysis of variance (ANOVA). To compare the differences between the treatments, Duncan's Multiple Range Test was used. The significant difference was set at $P<0.05$ and the data are represented as mean ± SEM. To estimate the suitable concentration of nevadensin for the cultivation of hybrid catfish, the second-order polynomial regression analysis was used (Yossa & Verdegem, 2015).

Results and discussion

1. Growth parameters and survival

As shown in Table 2, fish fed on 30 and 50 mg nevadensin/kg diet showed a significant increase ($P<0.05$) in the final weight, WG, SGR, ADG, and FI compared to the control. The final length of the treated fish was significantly higher ($P<0.05$) than that of the control fish. There was a significant improvement ($P<0.05$) in the FCR in fish fed nevadensin at 30 and 50 mg/kg compared with the control and those fed 10 mg nevadensin/kg diet. The SR levels were not significantly different ($P>0.05$) in all treated groups compared to the control and ranged from 99.16 to 100%. By using the second-order polynomial regression analysis on the FW ($y = -0.007x^2 + 0.436x + 21.755$, $R^2 = 0.133$, $P = 0.040$) and WG ($y = -0.007x^2 + 0.429x + 16.652$, $R^2 = 0.154$, $P = 0.023$), the optimal dosage of nevadensin for cultured catfish was found to be between 30.64 and 31.14 mg/kg diet (Fig. 3).

Table 2 Growth parameters and survival of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
IW (g)^{ns}	5.44 ± 0.17	6.22 ± 0.22	5.66 ± 0.16	5.11 ± 0.11
FW (g)	26.11 ± 1.84^{b}	28.55 ± 1.76^{ab}	32.66 ± 1.90^{a}	31.88 ± 1.67^{a}
IL (cm)^{ns}	8.77 ± 0.14	9.22 ± 0.12	8.88 ± 0.20	8.77 ± 0.16
FL (cm)	12.11 ± 0.77^{b}	16.22 ± 0.47^{a}	17.11 ± 0.51^{a}	17.22 ± 0.37^{a}
WG (g)	20.66 ± 1.78^{b}	22.33 ± 1.64^{ab}	27.00 ± 2.04^{a}	26.27 ± 1.68^{a}
SGR (%/day)	2.82 ± 0.11^{b}	2.75 ± 0.10^{b}	3.14 ± 0.15^{a}	3.28 ± 0.09^{a}
ADG (g/day)	0.36 ± 0.03^{b}	0.39 ± 0.02^{b}	0.48 ± 0.03^{a}	0.47 ± 0.03^{a}
FI (g/fish)	35.41 ± 1.02^{b}	37.14 ± 1.04^{ab}	38.44 ± 1.06^{a}	38.65 ± 1.01^{a}
FCR	1.72 ± 0.07^{a}	1.65 ± 0.09^{a}	1.42 ± 0.05^{b}	1.47 ± 0.08^{b}
SR (%)^{ns}	100.00 ± 0.00	100.00 ± 0.00	99.16 ± 0.83	100 ± 0.00

Remark: Data are represented as mean ± SEM. Different superscripts (^{a,b}) within a row are significantly different ($P<0.05$). Superscript ^{ns} indicates no statistical difference ($P>0.05$). IW = initial weight (g), FW = final weight (g), IL = initial length (cm), FL = final length (cm), WG = weight gain (g), SGR = specific growth rate (%/day), ADG = average daily gain (g/day), FI = feed intake (g/fish), FCR = feed conversion ratio, SR = survival rate (%).

The results of this study demonstrated that hybrid catfish fed different nevadensin diets showed a significant increase in growth performance, feed intake, and feed utilization compared to the control. No effects on SR values were observed in the tested fish compared to the control fish. Previous studies have shown that increased growth parameters of fish fed the diets supplemented with functional feed additives could be attributed to the improvement of nutrient digestion and absorption (Adel et al., 2015; Zheng et al., 2015). Additionally, the enhancement in feed intake and feed utilization efficiency by the feed additives supplemented diets could be associated with an increase of feed attraction and palatability in fish (Munglue, 2015; Reverter et al., 2014; Van Hai, 2015). Such reports suggested that medicinal

plants and their isolated phytochemicals have been reported to have positive effects on the growth and general health of several species of aquatic animals (Hoseini et al., 2018; Jahazi et al., 2020; Jia et al., 2019; Pês et al., 2015, 2016). It was demonstrated that juvenile northern snakehead fish (*Channa argus*) fed the diets incorporated with flavonoids from *Allium mongolicum* significantly improved growth rate, immunity, and antioxidant responses compared with the control (Li et al., 2019). Additionally, Nile tilapia (*Oreochromis niloticus*) fed dietary limonine showed a marked increase in FW and %WG (Aanyu et al., 2018). Jahazi et al. (2020) stated that common carp (*Cyprinus carpio*) fed the diets containing polyphenols extracted from chestnut and olive mill wastewater showed a significant improvement in growth indices including FW, SGR, WG and FCR compared with the control. The mechanisms of growth-promoting effects of herbal plants or their derivatives in fish have not yet been elucidated. It was speculated that phytochemicals could improve the expression of specific genes to modulate growth, feeding behavior, appetite regulation, digestive enzymes, nutrient digestion and metabolism of the fish including growth hormone, insulin growth factor (IGFs)-I and -II (Aanyu et al., 2018; Ahmadifar et al., 2019; Munglue, 2015; Sruthi et al., 2018). In this present research, fish that were fed with nevadensin diets showed significant increases in growth and feed utilization efficiency. As mentioned above, the growth-promoting property of nevadensin may be attributed to the improvement of feed palatability, feed intake, nutrient digestion and absorption as well as the modulation of some key genes (Ahmadifar et al., 2019; Jahazi et al., 2020; Munglue et al., 2019b; Sruthi et al., 2018). However, further research is required to clarify this hypothesis.

2. Organosomatic indices

Organosomatic indices of fish from different treatments are demonstrated in Table 3. It was found that HSI significantly increased ($P<0.05$) in fish fed 30 mg nevadensin/kg diet compared to the control and those fed 10 mg nevadensin/kg diet. There was no significant difference ($P>0.05$) in the HSI of fish fed nevadensin at 50 mg/kg diet when compared to the control. Additionally, ISI, GSI, CSI RSI and SSI did not differ significantly among the treatments.

Organosomatic indices are the indicators of nutritional and general health conditions in fish which can be calculated by the ratios of organs to body weight (Dekić et al., 2016). Studies of organosomatic indices in this research indicated that HSI of fish fed nevadensin at 30 mg/kg diet was significantly higher than that of the control fish. However, fish fed nevadensin supplemented diets did not show significant differences in ISI, CSI, RSI and SSI. The improvement in HSI could be due to increased hypertrophy and hyperplasia of hepatocyte, through improved cell metabolism, cell division and cell proliferation by nevadensin supplemented diet (Anderson et al., 1988; Strüssmann & Takashima, 1990). Thus, this could be the reason for an increase in the relative weight of the liver of fish fed nevadensin-enriched diets observed in this research.

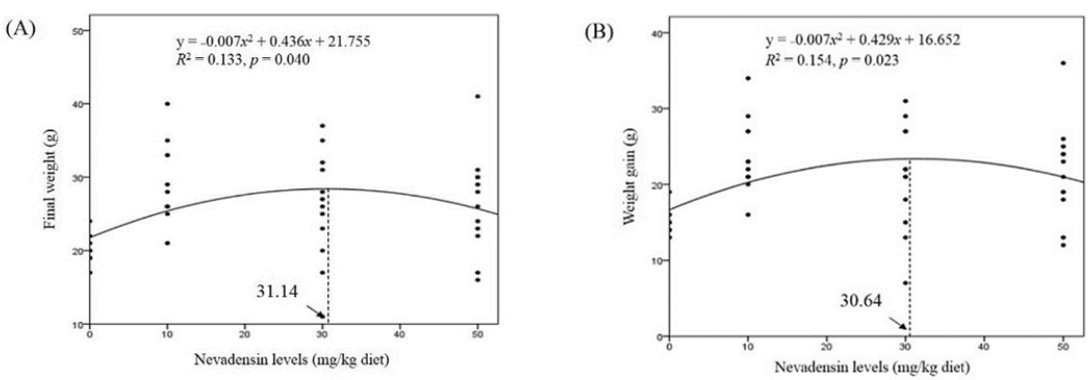

Fig. 3 The second-order polynomial regression analysis on (A) final weight (g) and (B) weight gain (g) of hybrid catfish fed the diets containing nevadensin at 0 (control), 10, 30, and 50 mg/kg diet for 8 weeks

Table 3 Organ somatic indices of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
HSI (%)	1.37 ± 0.05^{b}	1.43 ± 0.07^{b}	1.79 ± 0.01^{a}	1.61 ± 0.08^{ab}
ISI (%)^{ns}	2.70 ± 0.16	2.09 ± 0.25	2.24 ± 0.32	2.13 ± 0.221
GSI (%)^{ns}	4.20 ± 0.15	4.31 ± 0.50	4.63 ± 0.01	5.42 ± 0.18
CSI (%)^{ns}	0.14 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
RSI (%)^{ns}	0.48 ± 0.04	0.50 ± 0.03	0.43 ± 0.01	0.52 ± 0.02
SSI (%)^{ns}	0.32 ± 0.02	0.30 ± 0.28	0.32 ± 0.02	0.31 ± 0.12

Remark: Data are represented as mean±SEM. Different superscripts (^{a,b}) within a row are significantly different ($P<0.05$). Superscript^{ns} indicates no statistical difference ($P>0.05$). HSI = hepatosomatic index (%), ISI = intestinosomatic index (%), GSI = gill somatic index (%), CSI = cardiosomatic index (%), RSI = renosomatic index (%), SSI = splenosomatic index (%).

3. Intestinal macromorphology

The effects of different nevadensin diets on intestinal macromorphology of hybrid catfish are summarized in Table 4.

In the proximal part of the intestines (Fig. 4, A-D), villi heights of fish fed on diets supplemented with 10 to 50 mg nevadensin were significantly increased ($P<0.05$) when compared with the control. The total muscular thickness and inner circulatory muscle thickness were significantly enhanced in fish fed on a diet of 50 mg nevadensin compared with the control group and those fed with 10 and 30 mg levels of nevadensin. The outer longitudinal muscle thicknesses were significantly improved in fish fed diets supplemented with nevadensin at10 and 50 mg/kg in comparison to the control and fish fed with nevadensin at 30 mg/kg diet. Decreased villi widths were noticed in fish fed diets supplemented with 10 and 30 mg nevadensin compared with the control.

In the middle part of the intestines (Fig. 4, E-H), a significant increase in villi height was observed in fish fed nevadensin at 50 mg/kg diet compared with the other groups. Significant decreases in villi width and total muscular thickness were found in fish fed 30 mg nevadensin/kg diet compared to the control. The outer longitudinal muscle thickness was significantly improved in fish fed with a diet of 10 mg nevadensin compared to other treatments. However, the inner circulatory muscle thicknesses of fish fed nevadensin containing diets were similar to the control ($P>0.05$).

In the distal part of the intestines (Fig. 4, I-L), villi heights were significantly improved in all experimental groups compared with the control group. The total muscular thickness and inner circulatory muscle thickness were significantly enhanced in the fish fed with the diet containing 30 mg nevadensin compared to other treatments. Decreased villi widths were detected in fish fed nevadensin at 10 or 50 mg/kg diet compared with the control and those fed 30 mg nevadensin/kg diet. The outer longitudinal muscle thickness reduced significantly in fish fed nevadensin at 50 mg/kg diet compared to the control and those fed with 10 or 30 mg nevadensin/kg diet.

Table 4 Macromorphology of the intestines of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
Proximal portion				
Villi height (µm)	1418.76 ± 85.14^{c}	2414.42 ± 129.62^{a}	2641.12 ± 148.09^{a}	1931.40 ± 71.64^{b}
Villi width (µm)	627.03 ± 43.42^{a}	466.06 ± 28.37^{b}	479.06 ± 54.54^{b}	553.20 ± 49.84^{ab}
Total muscular thickness (µm)	204.78 ± 27.62^{b}	203.83 ± 15.92^{b}	197.92 ± 12.53^{b}	378.41 ± 43.93^{a}
Inner circulatory muscle thickness (µm)	124.38 ± 9.81^{b}	156.37 ± 12.53^{b}	157.22 ± 13.53^{b}	286.07 ± 33.14^{a}
Outer longitudinal muscle thickness (µm)	38.66 ± 3.68^{c}	47.46 ± 4.67^{b}	40.69 ± 3.58^{c}	92.33 ± 11.20^{a}
Middle portion				
Villi height (µm)	1345.54 ± 63.38^{b}	1343.54 ± 79.70^{b}	1150.25 ± 97.84^{b}	1429.32 ± 80.74^{a}
Villi width (µm)	551.95 ± 39.80^{a}	469.88 ± 31.09^{ab}	436.26 ± 20.41^{b}	502.57 ± 37.41^{ab}
Total muscular thickness (µm)	181.40 ± 20.86^{a}	194.17 ± 15.24^{a}	133.49 ± 19.40^{b}	160.18 ± 10.36^{ab}
Inner circulatory muscle thickness (µm)^{ns}	129.92 ± 18.15	124.04 ± 8.49	96.63 ± 14.12	109.37 ± 8.76
Outer longitudinal muscle thickness (µm)	51.47 ± 4.17^{b}	$70.12 \pm 8.07a$	36.87 ± 5.66^{b}	50.81 ± 4.12^{b}
Distal portion				
Villi height (µm)	1004.14 ± 50.79^{c}	1209.03 ± 43.94^{b}	1704.24 ± 110.05^{a}	1226.31 ± 53.77^{b}
Villi width (µm)	676.10 ± 41.62^{a}	440.86 ± 33.54^{b}	580.86 ± 68.96^{a}	522.48 ± 31.38^{b}
Total muscular thickness (µm)	162.03 ± 16.71^{b}	168.29 ± 22.89^{b}	215.37 ± 34.52^{a}	155.47 ± 12.75^{b}
Inner circulatory muscle thickness (µm)	121.55 ± 15.32^{b}	124.35 ± 18.36^{b}	161.99 ± 27.69^{a}	125.94 ± 12.77^{b}
Outer longitudinal muscle thickness (µm)	40.47 ± 3.78^{a}	43.94 ± 5.18^{a}	53.38 ± 8.35^{a}	29.53 ± 2.83^{b}

Remark: Data are represented as mean ± SEM. Different superscripts ($^{a-c}$) within a row are significantly different ($P<0.05$). Superscript^{ns} indicates no statistical difference ($P>0.05$).

It is well established that villi height is directly related to the abilities of the digestion and absorption of the gut (Boonanuntanasarn et al., 2018; Caballero et al., 2003). Intestinal muscular thickness plays a functional role in the maintenance of intestinal movement to support nutrient absorption as well as water reabsorption (Zhu et al., 2012). In this study, the application of nevadensin to the diets caused a significant increase in villi height and muscular thickness in fish intestines. These results are in agreement with the report of Ferreira et al. (2017) who found that the thickness of the muscular layer of *Astyanax aff. bimaculatus* fed the diets supplemented turmeric (*Curcuma longa*) significantly increased when compared to the control. The mechanisms

underlying of positive effects of dietary nevadensin on intestinal villi height and muscular thickness in this research are unclear. However, it is believed that nevadensin could regulate the expression of specific genes to modulate intestinal cell division and proliferation (Villasante et al., 2016). Additionally, nevadensin may induce the release of IGFs-I and II to enhance myogenesis in the intestine, leading to an increase in the muscular thickness (Crosnier et al., 2006). Thus, the findings of current work suggest that increased intestinal villi height and muscular thickness observed in fish fed the nevadensin diets could support feed digestion and water reabsorption in the gastrointestinal tract, increasing growth and nutrient utilization in fish (Munglue et al., 2019b; Zheng et al., 2015).

4. Intestinal micromorphology

The effects of dietary supplementation of nevadensin on intestinal micromorphology of hybrid catfish are demonstrated in Table 5.

In the proximal portion of the intestines, significant decreases ($P<0.05$) in total enterocyte heights and nucleus heights were obtained from fish received the diets supplemented with nevadensin compared to the control. Subnucleus heights in fish fed with the diets containing nevadensin were significantly higher than those fed the basal diet. A significant increase in microvilli heights was detected in fish fed diets supplemented with 30 or 50 mg of nevadensin. The goblet cell number of fish fed with 10 mg nevadensin/kg diet was significantly lower than fish fed with 30 mg nevadensin/kg diet. However, supranucleus heights and nucleus widths did not differ significantly ($P>0.05$) among the experimental groups.

In the middle portion of the intestines, total enterocyte heights and supranucleus heights significantly declined in fish fed with the diets containing nevadensin compared with fish fed the basal diet. A significant decrease in nucleus height was observed in fish fed the diet containing 10 mg nevadensin compared to the other treatments. Those fish fed 10 or 50 mg nevadensin/kg

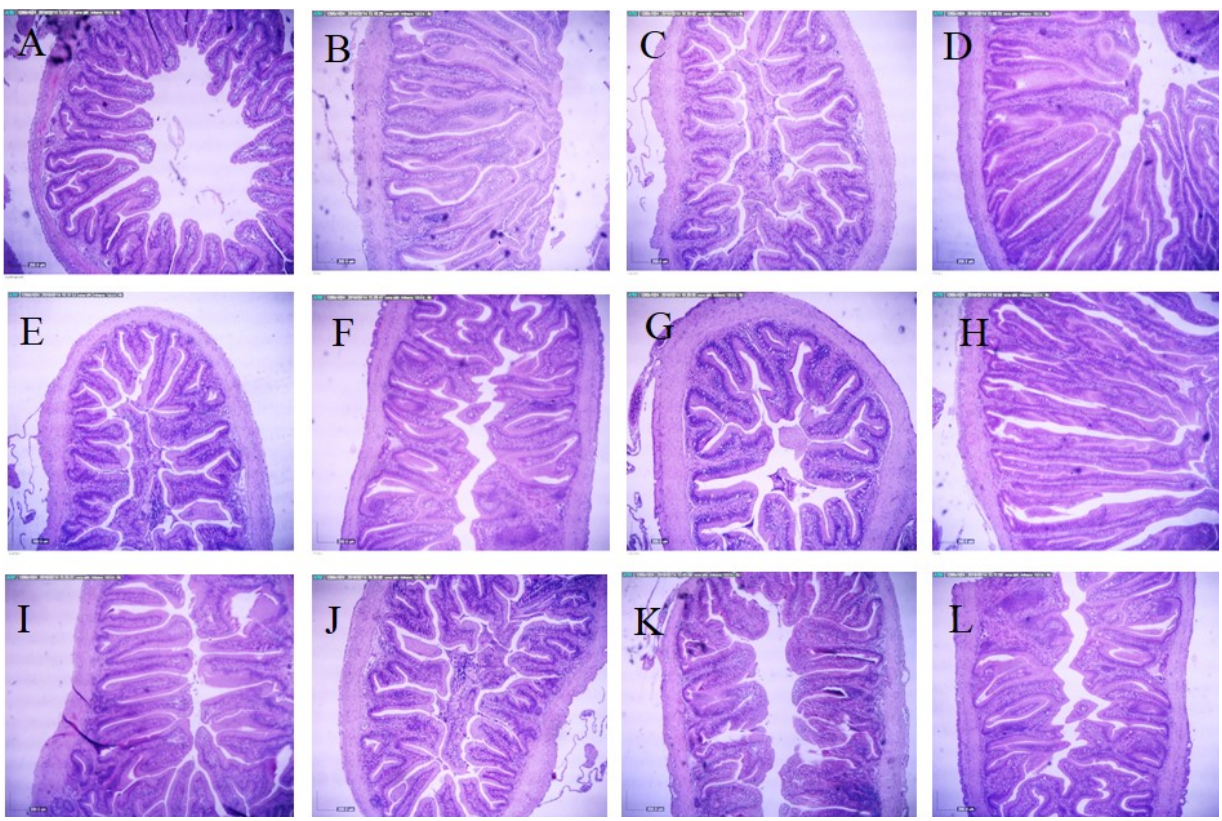

Fig. 4 Proximal (A - D), middle (E - H), and distal (I - L) portions of the intestines of hybrid catfish fed the diets containing nevadensin at 0 (A, E, and I), 10 (B, F, and J), 30 (C, G, and K), and 50 (D, H, and L) mg/kg diet for 8 weeks. Scale bar = 200 µm

diet produced a significant increase in goblet cells compared with the control. Significant increases in microvilli heights were observed in fish fed with nevadensin containing diets. Subnucleus heights and nucleus widths did not affect by nevadensin supplementation.

In the distal portion of the intestines, total enterocyte heights were significantly decreased in fish fed nevadensin containing diets compared with the control. A significant reduction of supranucleus height was found in fish fed 10 or 30 mg nevadensin/kg diet compared with other diets. Subnucleus height decreased significantly in fish fed 30 mg nevadensin diet compared to fish fed 50 mg nevadensin diet. The nucleus height in fish fed 10 mg nevadensin diet was significantly lower than that in fish fed the control diet. The goblet cell number and microvilli height increased significantly in the experimental groups compared to the control. Additionally, no significant differences were noted in the nucleus widths among the treatments.

Enterocyte characteristics, microvilli, and goblet cell could be used to demonstrate the physiological functions of fish intestines. Previous reports have shown that starvation and feed ingredients can affect the micromorphology of the intestines (Rašković et al., 2011; Savic et al., 2012). The longer enterocyte and microvilli would represent the effective nutrient absorption processes that occurred in the intestines, leading to the higher growth and feed efficiency of fish (Fonseca-Madrigal et al., 2006; Ostaszewska et al., 2005). Mucins, high-molecular-weight glycoproteins produced by goblet cell, play a key role in osmoregulation, protection of intestinal epithelial cells from mechanical and chemical damages and modulation of intestinal absorption processes (Kim & Ho, 2010; Kozarić et al., 2007). In this study, significant increases in goblet cell number, subnucleus height, and microvilli height were found in the tested fish. In contrast, enterocyte height, supranucleus height, and nucleus height decreased significantly in the experimental groups compared with the control. These findings were consistent with the report that lasia supplemented diets produced a marked decrease of enterocyte height and caused a significant increase of microvilli height in hybrid catfish (Munglue et al., 2019a). Similarly, sharpsnout seabream (*Diplodus puntazzo*) fed soybean meal-based diets containing mannanoligo-saccharides and inulin for 114 days significantly decreased enterocyte height values both in the proximal and distal part of intestines (Ferrara et al., 2015). As mentioned above, the increases in enterocyte height, microvilli height, and goblet cell numbers are related to better growth and feed utilization efficiency of fish (Akter et al., 2016; Boonanuntanasarn et al., 2018; Poleksić et al., 2014). Increased goblet cell numbers may in part be related to the enhancement of cell proliferation, cell differentiation, and cell division which might be modulated by nevadensin to improve intestinal functions (Ostaszewska et al., 2005). The longer enterocyte and microvilli would indicate the higher absorptive area of the intestines (Akter et al., 2016; Poleksić et al., 2014). In this current work, the decline in enterocyte height did not relate to the growth observed in fish fed nevadensin diets. Thus, it is hypothesized that other specific factors that were modulated by nevadensin would also have positive effects on the growth of hybrid catfish (Munglue et al., 2019a).

Table 5 Micromorphology of the intestines of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
Proximal portion				
Total enterocyte height (μm)	69.63 ± 4.57^{a}	40.74 ± 2.06^{c}	53.13 ± 1.63^{b}	47.22 ± 1.78^{bc}
Supranucleus height (μm)^{ns}	26.78 ± 2.44	20.83 ± 1.56	21.51 ± 2.41	27.73 ± 2.15
Subnucleus height (μm)	4.44 ± 0.26^{b}	12.80 ± 0.89^{a}	12.43 ± 0.65a	11.28 ± 0.90^{a}
Nucleus height (μm)	8.76 ± 0.27^{a}	5.37 ± 0.26^{c}	6.39 ± 0.41^{bc}	6.98 ± 0.46^{b}
Nucleus width (μm)^{ns}	11.49 ± 0.28	11.07 ± 0.26	10.33 ± 0.40	11.43 ± 0.21
Goblet cell number	8.00 ± 2.59^{ab}	6.33 ± 0.66^{b}	9.00 ± 0.64^{a}	8.86 ± 1.15^{ab}
Microvilli height (μm)	3.20 ± 0.26^{c}	4.96 ± 0.13^{bc}	4.60 ± 0.07^{b}	5.28 ± 0.14^{a}
Middle portion				
Total enterocyte height (μm)	72.82 ± 5.00^{a}	43.28 ± 2.15^{b}	46.43 ± 2.11^{b}	52.01 ± 2.08^{b}
Supranucleus height (μm)	31.47 ± 2.40^{a}	19.35 ± 1.54^{b}	19.15 ± 1.06^{b}	21.16 ± 0.87^{b}
Subnucleus height (μm)^{ns}	4.27 ± 0.33	4.39 ± 0.33	4.10 ± 0.42	3.79 ± 0.26
Nucleus height (μm)	7.58 ± 0.29^{a}	4.67 ± 0.35^{b}	6.35 ± 0.56^{a}	8.33 ± 0.75^{a}
Nucleus width (μm)^{ns}	11.74 ± 0.54	12.42 ± 0.66	10.39 ± 0.46	11.53 ± 0.36
Goblet cell number	8.86 ± 0.64^{b}	13.33 ± 1.52^{a}	11.40 ± 1.33^{ab}	12.20 ± 0.75^{a}
Microvilli height (μm)	3.12 ± 0.35^{b}	5.43 ± 0.10^{a}	5.20 ± 0.20^{a}	5.10 ± 0.18^{a}
Distal portion				
Total enterocyte height (μm)	96.65 ± 6.63^{a}	66.00 ± 4.12^{b}	69.09 ± 4.41^{b}	70.57 ± 5.68^{b}
Supranucleus height (μm)	43.10 ± 5.19^{a}	24.47 ± 1.44^{b}	30.76 ± 2.84^{b}	37.28 ± 4.46^{a}
Subnucleus height (μm)	4.13 ± 0.22^{ab}	4.16 ± 0.32^{ab}	4.67 ± 0.34^{a}	3.79 ± 0.16^{b}
Nucleus height (μm)	7.83 ± 0.27^{a}	6.63 ± 0.23^{b}	7.45 ± 0.46^{ab}	7.64 ± 0.21^{ab}
Nucleus width (μm)^{ns}	10.53 ± 0.62	10.40 ± 0.52	11.02 ± 0.53	11.07 ± 0.44
Goblet cell number	8.00 ± 0.66^{c}	12.04 ± 1.16^{b}	15.66 ± 1.57^{a}	13.80 ± 1.43^{ab}
Microvilli height (μm)	3.40 ± 0.16^{b}	4.83 ± 0.30^{a}	4.68 ± 0.30^{a}	4.63 ± 0.09^{a}

Remark: Data are represented as mean ± SEM. Different superscripts ($^{a-c}$) within a row are significantly different ($P<0.05$). Superscript ^{ns} indicates no statistical difference ($P>0.05$).

5. Hematology

The effects of nevadensin containing diets on the hematology of hybrid catfish are shown in Table 6. The levels of RBC and WBC of fish fed the diets containing nevadensin were significantly higher ($P<0.05$) than those of fish fed with the basal diet. HGB increased significantly in fish fed 50 mg nevadensin/kg diet

compared with the control, but it was not significantly different (P<0.05) from that of fish fed 10 or 30 mg nevadensin/kg diet. Additionally, fish fed a diet with 50 mg nevadensin showed the highest HCT level. The highest lymphocyte was noticed in fish fed with the diet containing 10 mg nevadensin/kg. No significant differences (P>0.05) were detected in PLT, MCV, MCH, MCHC, neutrophils, monocytes, basophils and eosinophils among the treatments.

Hematological indices can be useful for the evaluation of the general health status and well-being of fish (Campbell & Ellis, 2007; Clauss et al., 2008). Several intrinsic and extrinsic factors can affect cell morphology and the quantitative levels of the blood cells (Burgos-Aceves et al., 2019). The present study, it was demonstrated that the diets supplemented with nevadensin produced a significant increase in RBC, WBC, HGB, HCT and lymphocyte compared with the control diet. Many reports have been indicated the positive effects of medicinal plants and their derivative compounds on the hematological values of fish (Abdel-Tawwab et al., 2018; Awad & Awaad, 2017; Van Hai, 2015). Adel et al. (2015) noticed that fry Caspian white fish (*Rutilus frisii kutum*) fed peppermint-enriched diets for 8 weeks significantly enhanced RBC, WBC, HCT, HGB, and neutrophil compared with the control. Also, Giri et al. (2017) showed that RBC and WBC of *Labeo rohita* fed the diets mixed with *Hybanthus enneaspermus* aqueous extract for 6 weeks were significantly increased, while neutrophil and lymphocyte were significantly decreased compared with the control. Improved RBC count and HGB by dietary nevadensin may enhance the oxygen-carrying capacity and the response of fish against physiological stresses (Gabriel et al., 2019). Increased WBC and lymphocyte levels in fish fed dietary nevadensin could be demonstrated the enhancement of non-specific immune responses by this compound (Adel et al., 2015; Awad & Awaad, 2017). It is suggested that dietary nevadensin could help to protect fish from several infectious diseases and stressful conditions (Reverter et al., 2014). It is assumed that nevadensin may stimulate hematopoietic stem cells in hematopoietic organs to enhance RBC and WBC in the fish as demonstrated in the report of Gabriel et al. (2019). Thus, increased levels of WBC and lymphocyte in fish fed nevadensin supplemented diets would indicate the immunostimulatory potential of this natural compound.

Table 6 Hematological values of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
RBC ($\times 10^6$ cell/mm^3)	1.76 ± 0.40^b	2.74 ± 0.10^a	2.50 ± 0.09^a	2.64 ± 0.17^a
WBC ($\times 10^4$ cell/mm^3)	36.80 ± 3.98^b	49.16 ± 2.78^a	52.18 ± 3.85^a	48.76 ± 3.92^a
HGB (g/dL)	9.12 ± 1.49^b	11.70 ± 0.44^{ab}	11.12 ± 0.35^{ab}	12.98 ± 0.83^a
HCT (%)	25.66 ± 0.33^b	23.66 ± 0.88^b	22.33 ± 1.85^b	30.00 ± 3.05^a
PLT ($\times 10^3$ cell/µL)^{ns}	32.60 ± 4.40	49.00 ± 11.93	40.00 ± 9.11	46.75 ± 7.72
MCV (fL)^{ns}	134.38 ± 5.90	119.14 ± 2.30	122.30 ± 4.68	126.98 ± 6.12
MCH (pg)^{ns}	46.68 ± 0.49	42.60 ± 0.83	44.32 ± 0.51	49.08 ± 0.29
MCHC (g/dL)^{ns}	35.06 ± 1.45	35.84 ± 1.02	36.50 ± 1.26	39.00 ± 1.69
Lymphocyte (%)	64.33 ± 4.63^b	81.66 ± 2.02^a	65.66 ± 7.68^b	63.33 ± 2.33^b
Neutrophil (%)^{ns}	10.00 ± 0.00	3.50 ± 1.53	8.66 ± 6.02	12.33 ± 2.51
Monocyte (%)^{ns}	13.33 ± 8.08	5.50 ± 2.12	9.66 ± 5.13	13.33 ± 6.65
Basophil (%)^{ns}	8.00 ± 1.52	8.00 ± 1.15	10.66 ± 5.69	3.00 ± 1.00
Eosinophil (%)^{ns}	4.66 ± 2.72	4.33 ± 2.33	8.00 ± 0.00	8.00 ± 2.51

Remark: Data are represented as mean ± SEM. Different superscripts (^{a,b}) within a row are significantly different (P<0.05).
Superscript ^{ns} indicates no statistical difference (P>0.05). RBC = red blood cell ($\times 10^6$ cell/mm^3), WBC = white blood cell ($\times 10^4$ cell/mm^3), HGB = hemoglobin (g/dL), HCT = hematocrit (%), PLT = platelet ($\times 10^3$ cell/µL), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL).

6. Serum biochemistry

As shown in Table 7, dietary supplementation with nevadensin had no effects (P>0.05) on ALP, AST, albumin, creatinine, triglyceride, total protein and globulin levels in hybrid catfish compared with the practical diet. Fish fed the diets with nevadensin tended to have lower glucose levels compared to the fish fed the basal diet, although this effect did not reach statistical significance.

Serum biochemical values are very useful for monitoring stress, nutritional imbalance and the health status of fish (Yang & Chen, 2003; Zhai et al., 2016). In this study, it was found that nevadensin enriched-diets had no significant effects on the levels of AST, ALP, creatinine, glucose, triglyceride, total protein, albumin and globulin in hybrid catfish. Enzyme activities of AST and ALP are directly related to hepatic, gill and muscle functions (Yang & Chen, 2003). An increase in AST and ALP levels may indicate hepatic injuries due to malnutrition, toxins, infectious diseases and stress (Coppo et al., 2003). Creatinine is mainly a waste product produced by the breakdown of muscle cells and is generally used as an indicator of renal functions (Abdel-Tawwab et al., 2017; Coppo et al., 2003). Increased creatinine levels could indicate kidney injury and infectious diseases (Mutlu et al., 2015). In this study, dietary nevadensin did not produce any change in the levels of AST, ALP and creatinine in hybrid catfish. It is

suggested that nevadensin had no effects on the structural and functional integrity of the kidney, liver and muscle cells. Increased serum glucose level is an indicator of the environmental stress in fish (Adham et al., 2002; Güllü et al., 2016). In this present study, serum glucose levels tend to decrease in fish fed nevadensin diets compared with the control. This result suggested that nevadensin may have hypoglycemic action due to increased glucose uptake and glycogenesis in certain peripheral tissues of fish (Sruthi et al., 2018; Wang et al., 2018). It was postulated that serum triglyceride is directly correlated with the presence of lipid peroxidation and reactive oxygen species in fish (Gabriel et al., 2015). Increased triglyceride levels may indicate the prevalence of higher lipid peroxidation and oxidative stress. In this study, it was found that the levels of triglyceride in fish fed the diets containing nevadensin were similar to the control. Thus, it seems that nevadensin could have anti-lipid peroxidation and anti-inflammatory effects in fish (Gabriel et al., 2015; Reddy et al., 1991). The levels of total protein, albumin and globulin are associated with the innate immune response in fish (Güllü et al., 2016). The results showed that no significant differences in total protein, albumin and globulin were detected in fish fed dietary nevadensin. Therefore, it could be suggested that nevadensin supplementations at 10 to 50 mg/kg feed did not affect serum levels of total protein, albumin and globulin in hybrid catfish.

Table 7 Blood biochemical values of hybrid catfish fed the diets supplemented with different levels of nevadensin for 8 weeks

Parameters	Nevadensin levels in the experimental diets (mg/kg diet)			
	0	10	30	50
AST (U/L)^{ns}	170.09 ± 34.17	171.85 ± 46.26	105.87 ± 14.82	163.31 ± 12.15
ALP (U/L)^{ns}	31.73 ± 4.66	35.25 ± 4.43	37.02 ± 8.14	26.44 ± 3.05
Creatinine (mg/dl)^{ns}	0.95 ± 0.43	0.70 ± 0.10	1.04 ± 0.34	0.54 ± 0.14
Glucose (mg/dl)^{ns}	168.32 ± 42.22	163.39 ± 25.28	122.85 ± 11.95	123.33 ± 20.41
Triglycerides (mg/dl)^{ns}	176.92 ± 47.57	198.90 ± 16.31	285.37 ± 76.44	191.64 ± 9.03
Total protein (mg/dl)^{ns}	4.04 ± 0.18	3.36 ± 0.93	4.28 ± 0.31	2.65 ± 0.63
Albumin (mg/dl)^{ns}	1.59 ± 0.15	1.44 ± 0.03	1.41 ± 0.20	1.46 ± 0.07
Globulin (mg/dl)^{ns}	2.45 ± 0.34	1.91 ± 0.94	2.87 ± 0.10	1.18 ± 0.23

Remark: Data are represented as mean ± SEM. Superscript ^{ns} indicates no statistical difference (P>0.05). AST = aspartate transaminase (U/L), ALP = alkaline phosphatase (U/L).

Conclusion

To conclude, this research exhibited that dietary nevadensin produced the improvement in growth performance, feed intake, feed utilization, intestinal histology and hematology of hybrid catfish. By using the second-order polynomial regression analysis, the recommended dose of nevadensin for the cultivation of hybrid catfish observed in this present study was found to be between 30.64 and 31.14 mg/kg diet.

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Assessment of Water Quality in the Stream Flows into the Krasiow Dam based on Hydropsychid Larvae (Insecta, Trichoptera)

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Abstract

The use of altered gill morphology in hydropsychid larvae (Hydropsychidae: Trichoptera) as water quality biomonitoring was conducted in streams flows into Krasiow Dam, Dan Chang district, Suphan Buri Province, Thailand. Samples were collected over a period of one year January to December 2019. In total, 5,583 individual hydropsychid larvae belonging to four genera (*Amphipsyche meridiana, Macrostemum indistinctum, Cheumatopsyche* spp. and *Potamyia* spp.) were identified in this study. The percentage of individuals with at least some abnormality (HAI) and hydropsychid gill abnormality indice (HYI) was the highest in February and lowest in October. Canonical Correspondence Analysis (CCA) indicated the species *Amphipsyche meridiana* were positively correlated with water temperature, pH, and ammonia-nitrogen. Total dissolved solids were negatively associated with the *Cheumatopsyche* spp. and *Macrostemum indistinctum*. The outcomes of this study is expected that analyses of morphological deformations in tracheal gills could become a support for traditional physicochemical analyses of water quality or traditional biomonitoring.

Introduction

The net-spinning Hydropsychidae are one of the largest families of caddisflies (Trichoptera), with about 1,900 described adult species worldwide (Morse, 2017). Larvae of hydropsychids live in running waters and are generally collectors-filterers, although some species are predators of larval black flies (Merritt & Cummins, 1996). They usually construct a silken filter net at the entrance to their fixed tubular retreat (Wiggins, 1996). Larvae present a high ecological diversity and display a wide range of tolerance to different levels of pollution, which makes them very useful organisms in biological water quality monitoring programs (Resh, 1995). Due to their high abundance, wide distribution and important role in the ecology of the lotic ecosystem, the hydropsychid caddisflies have been increasingly utilized in the biomonitoring and impact assessment of the river (Vuori, 1995). Hydropsychid larvae have been used as sentinel organisms when measuring the environmental concentration levels of contaminants (Cain et al., 1992; Vuori, 1994; Clements & Kiffney, 1994) and also as monitoring and toxicity test organisms both in the laboratory and in the field (Vuori, 1995). In these studies, the individual, population and guild responses of the hydropsychid larvae have been found to be sensitive indicators of

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river pollution.

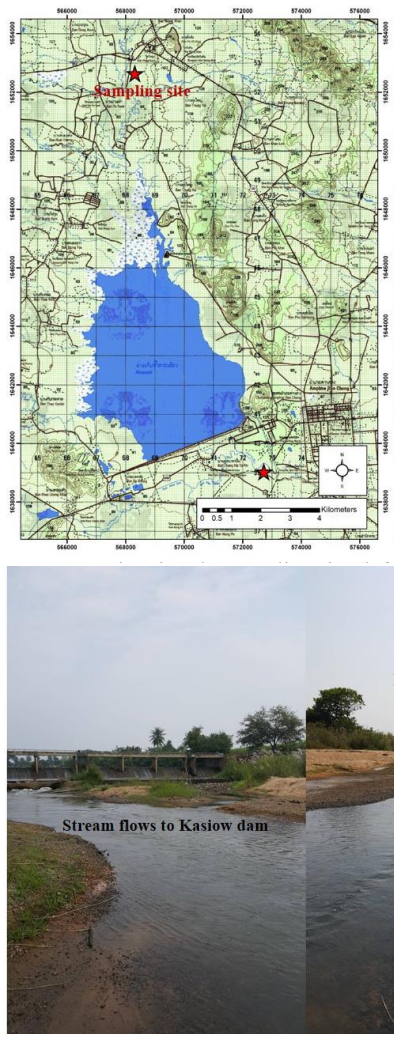
The assessment of the impact of pollution using individuals and populations of benthic invertebrates has a number of advantages over the traditional biomonitoring methods utilizing community structure of benthic invertebrates (Rosenberg & Resh, 1993). Although importance in describing the general state of the river ecosystem, the community derived parameters are robust, inconclusive and often insensitive to gradual environmental changes. Pollution, as a selective factor, mainly affects the mortality, growth and reproduction of individuals and populations. Hence, individual and population parameters are likely to be more sensitive and accurate indicators of increasing stress than parameters utilizing whole communities. Furthermore, while the objective criteria for the determination of healthy ecosystems and communities is lacking, the occurrence of unhealthy individuals is often easily distinguishable and can be linked to the effects at the population level (Forbes & Forbes, 1994). However, studies dealing only individual and population responses are unable to detect changes in such community level phenomena as species interactions and diversity. These changes may become apparent if a group of ecologically related species, is studied (Petersen, 1986).

Upon exposure to pollutants, some changes at the biochemical, cellular or tissue levels must precede changes at the higher levels of biological organization (Giesy & Graney, 1989). Morphological abnormalities in the tracheal gills and ion regulation organs, and anal papillae of hydropsychid larvae indicate cellular and tissue which leads to the impairment of the individual's respiratory or osmoregulatatory functions (Camargo, 1991; Vuori, 1994). Hence, the simultaneous study of the morphology, life cycle and species composition of the hydropsychid larvae may provide information on the impact of pollution at a cellular, individual and population level and even offer clues to possible mechanisms at the community level (Vuori, 1995). The aim of this study was to analyze the usefulness of hydropsychid larvae in evaluation of water quality in stream that surrounding by agricultural activities. Our intention was to find a relationship between gill morphological changes and level of water contamination in the studied streams. The outcome of this study is expected that analyses of morphological deformations in tracheal gills could become a support for traditional physicochemical analyses of water quality or traditional biomonitoring.

Materials and methods

1. Study area

The samples were conducted in the stream flows into the Kasiow Dam, Dan Chang district, Suphan Buri Province (14°56.859′ N, 099°38.118′ E, 81 m asl.) (Fig. 1). Kasiow stream, the stream that flows through Kasiow Dam, is a main branch of Thachin River, approximately 140 kilometers. The upstream is located between Khao La and Khao Yai in the north part of Ban Rai district, Uthai Thani Province. This stream flows pass the area in Dan Chang district, which the sugarcane plantation and sugar factories are located near the stream. In addition, fish cage farming are reared in the area of Kasiow Dam.



A

B

Fig. 1 Map showing the sampling site (A) of the stream flows (B) into Kasiow Dam in Dan Chang district, Suphan Buri Province, Thailand

2. Environmental parameters

In order to observe the relationship of caddisflies species occurrence to environmental parameters the following data were recorded for water body. Three replicates of selected physicochemical water quality parameters were recorded directly at the sampling site. Water temperature (°C) and dissolved oxygen (DO, mg/L) were measured in situ with HQ40D Portable Dissolved Oxygen and Conductivity/TDS Meter. The pH was measured by a pH-meter Waterproof Model Testr30. Water samples from each collecting period were stored in polyethylene bottles (500 mL). Nitrate-nitrogen (mg/L NO_3-N, ascorbic acid method), orthophosphate (mg/L PO_4^{3-}, cadmium reduction method) and ammonia-nitrogen (mg/L NH_3-N, the Nessler method) were measured by using the Hach DR/2000 spectrophotometer (APHA, 1992).

3. Sample collection and identification

The caddisfly larvae were collected from stream flows into Kasiow Dam during January to December 2019, except the month of April, May and June. In April, when the stream water was particularly warm, the caddisfly larvae were not found. In May and June during the storm season the stream water was very turbid. At each sampling period, semi-quantitative samples of caddisfly larvae from the different microhabitats (riffles, depositional zones and different types of vegetation) were collected. A D-frame (Merritt & Cummins, 1996) aquatic hand net (mesh sieve 250 μm) was used. The samples of the aquatic hand net were poured into white trays. Living caddisflies were sorted and transferred into properly-labelled plastic containers, preserved in 80% ethanol and brought to the laboratory for analysis.

In the laboratory, caddisfly larvae were sorted on a petri dish and were identified to the genus and species level using taxonomic keys by several authors (Wiggins, 1996; Dudgeon, 1999; Yule & Yong, 2004; Prommi, 2007). All the sorted samples were kept in properly-labelled vials containing 80% ethanol.

4. Data analysis

Structural changes in the hydropsychid gills were studied under a stereomicroscope and quantified using an ocular micrometer. Small and light pigmentation spots were considered as 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). One way ANOVA was used to determine the difference in physicochemical characteristics among each month. Canonical correspondence analysis (CCA) was applied to test the influence of environmental variables on the hydropsychid assemblages. Biological data were log (X+1) transformed prior to canonical correspondence analysis. A Monte Carlo permutation test with 998 random permutations was used to examine a statistical significance of the model determination. Multivariate analyses were performed by using PC-ORD ver.5 (McCune & Mefford, 2006).

Results and discussion

1. Physicochemical parameters of water quality

One-way ANOVA showed significant difference of all parameters such as water temperature, total dissolved solids (TDS), dissolved oxygen (DO), pH, orthophosphate (PO_4^{2-}), ammonia-nitrogen (NH_3-N) and nitrate-nitrogen (NO_3-N) in all sampling occasions ($p<0.05$) (Table 1). The annual variations in environmental factors in the stream flows into Kasiow Dam for one years are shown in Table 1. The water temperature showed very wide range of fluctuation, (22.37±0.21 −39.5±1.15ºC). Also, the total dissolved solids showed very wide range of fluctuation, (95.93±1.05−385.50±0.35 mg/L). The higher values of TDS were the indicators of higher ionic concentrations, probably due to the high anthropogenic activities in the region and geological weathering conditions acquiring high concentrations of the dissolved minerals (Costello et al., 1984).

The local anthropogenic activities could be the discharges from intensive and prolonged agricultural activities and discharges from industrial and domestic wastes. Agricultural activities introduce ions and metals from fertilizers and other agrochemicals (Hamid et al., 2020). The DO showed very narrow range of fluctuation, (6.13±0.16−8.63±0.50 mg/L). The range of DO of the stream water flows into Kasiow Dam was found within the normal range (>2 to 6 mg/L) in the stream water and considered very good for most stream biotas (PCD, 2014). The pH of water was found slightly alkaline (7.47±0.15 −9.30±0.44). The pH range from 5.0 to 9.0 is permissible as per PCD (2014). Although pH usually has no direct impact to consumers, it is one of the most important operational water quality parameters.

Table 1 Mean ±SD water quality parameters of the stream flows into Kasiow Dam during January to December 2019.

Factor/ month	WT	DO	pH	TDS	NH_3-N	NO_3-N	PO_4^{3-}
Jan	27.67±0.31^{bc}	8.21±0.13^{c}	8.20±0.10^{bc}	228.67±0.58^{f}	0.08±0.04^{a}	4.13±0.06^{abd}	1.18±0.08^{d}
Feb	30.13±1.18^{de}	7.21±0.13^{b}	7.97±0.40^{abc}	111.67±11.50^{b}	0.69±0.34^{c}	7.20±0.17^{d}	2.25±0.15^{ef}
Mar	31.30±0.53^{e}	8.63±0.50^{c}	8.53±0.06^{c}	134.10±0.75^{c}	0.23±0.01^{ab}	4.10±0.17^{ab}	0.84±0.04^{cd}
Apr	39.5±1.15^{f}	6.88±0.65^{c}	7.87±0.1^{2ab}	95.93±1.05^{a}	0.72±0.04^{c}	-	0.76±0.25^{bcd}
May	-	-	-	-	-	-	-
Jun	-	-	-	-	-	-	-
Jul	29.13±0.06^{cd}	6.13±0.16^{a}	8.03±0.06^{abc}	136.70±3.91^{c}	0.22±0.13^{ab}	1.77±0.15^{abc}	0.26±0.27^{a}
Aug	29.87±0.06^{de}	6.65±0.07^{ab}	8.17±0.06^{bc}	162.57±0.41^{d}	0.20±0.03^{ab}	1.57±0.46^{ab}	0.17±0.07^{a}
Sep	29.10±0.53^{cd}	7.07±0.03^{c}	7.47±0.15^{a}	175.00±0.26^{e}	0.48±0.30^{bc}	5.63±4.21^{cd}	0.40±0.19^{abc}
Oct	31.60±1.08^{e}	6.89±0.07^{ab}	8.30±0.00^{bc}	245.67±2.52^{g}	0.26±0.01^{ab}	2.93±0.06^{abc}	0.67±0.10^{abcd}
Nov	22.37±0.21^{a}	6.99±0.02^{b}	9.30±0.44^{d}	385.50±0.35^{i}	0.15±0.03^{a}	3.70±0.00^{abcd}	1.82±0.39^{e}
Dec	26.80±0.17^{b}	7.06±0.04^{c}	8.43±0.06^{bc}	359.00±1.00^{h}	0.24±0.02^{ab}	4.40±0.62^{bcd}	2.43±0.06^{f}

Remark: Data shown with different letters are statisti cally significant at the $P < 0.05$ level

The orthophosphate concentration was highest (2.43±0.06 mg/L) in December and lowest in August (0.17±0.07 mg/L). The high concentration of phosphate in the stream may be due to land use management practices. Land use operations such as fertilizer application to promote agricultural products results in increased P release (Cummins & Farrell, 2003), and may result in increasing of P concentration in receiving water bodies (Lawniczak et al., 2016). Other important sources of phosphorus to freshwater are atmospheric precipitation, geochemical condition, and ground water. Concentration of nitrate-nitrogen and ammonia-nitrogen ranged from 1.57±0.46 to 7.20±0.17 mg/L and 0.08±0.04 to 0.72±0.04 mg/L, respectively. In natural aerobic water, most nitrogen occurs as nitrates in varying amount depending upon the nature of water shed, seasons, degree of pollution and the abundance of plankton (Maitland, 1978).

2. Biodiversity of hydropsychids larvae

In total 5583 individual hydropsychid larvae belonged to four genera. There are consists of the *Amphipsyche meridiana* which contains 2,442 individual (Fig. 2), followed by *Macrostemum indistinctum* (1438 individual, *Cheumatopsyche* spp. (1423 individual) and *Potamyia* spp. (280 individual) (Table 2). All four hydropsychid genera were found in each month, except in September. Only the *Potamyia* spp. were found, because of the stream water was slightly turbid and high water level during sampling.

3. Morphological abnormalities

Normal, undamaged gills are whitish and branching. According to Ratia et al. (2012), a gill tuft can be considered damaged if it is totally reduced or its basal or distal parts darkened, or if the gill tuft has dark spots on > 50 % of its branches. However, some studies consider only totally reduced gill tufts as damaged (Vuori, 1994; Vuori & Kukkonen 1996). Also darkening of the anal papillae of hydropsychid larvae has been observed as a response to metal exposure (Vuori, 1994).

Table 2 Number of normal and abnormal hydropsychid larvae collected in stream flows into Kasiow Dam during January to December 2019

Month	Taxa	Total	Total number	
			Normal	Abnormal
January	*Amphipsyche meridiana*	6	3	3
	Cheumatopsyche sp.	104	77	27
	Macrostemum indistinctum	33	24	9
	Potamyia sp.	217	183	34
February	*Amphipsyche meridiana*	89	24	65
	Cheumatopsyche sp.	33	14	19
	Macrostemum indistinctum	3	0	1
	Potamyia sp.	2	1	1
March	*Amphipsyche meridiana*	170	102	68
	Cheumatopsyche sp.	401	401	0
	Macrostemum indistinctum	130	113	17
July	*Amphipsyche meridiana*	670	489	181
	Cheumatopsyche sp.	11	4	6
	Macrostemum indistinctum	303	252	51
	Potamyia sp.	3	1	0
August	*Amphipsyche meridiana*	650	357	293
	Cheumatopsyche sp.	24	22	2
	Macrostemum indistinctum	270	180	90
	Potamyia sp.	4	2	2
September	*Potamyia* sp.	8	8	0
October	*Amphipsyche meridiana*	278	268	10
	Cheumatopsyche sp.	133	133	0
	Macrostemum indistinctum	39	36	3
	Potamyia sp.	33	33	0
November	*Amphipsyche meridiana*	156	22	134
	Cheumatopsyche sp.	191	151	13
	Macrostemum indistinctum	421	276	37
	Potamyia sp.	6	6	0
December	*Amphipsyche meridiana*	423	397	67
	Cheumatopsyche sp.	526	476	50
	Macrostemum indistinctum	239	215	34
	Potamyia sp.	7	7	0

The morphological abnormalities observed included the darkening and reduction of the tracheal gills (Fig. 2B) and the malformation of the whole gill tuffs (Fig. 2C) in all hydropsychids larvae collected. The wound-like malformations were also discovered in the abdominal segments of the larvae (Fig. 2D-F).

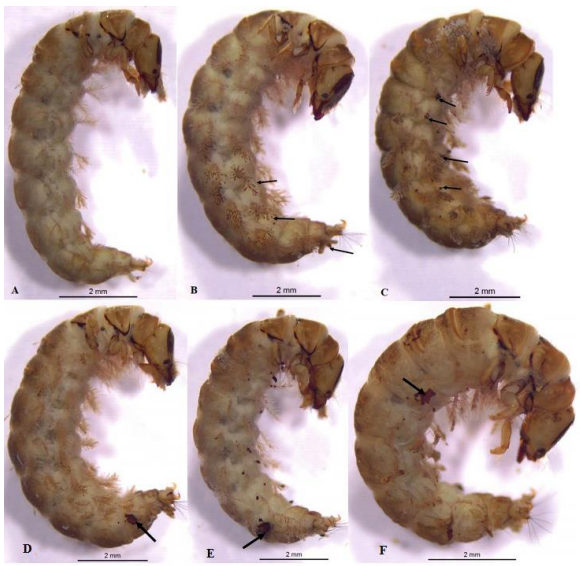


Fig. 2 Example of Hydropsychidae species, *Amphipsyche meridiana* that are found the most individual from this study. Gill tufts are the transparent branches on the ventral side (A), slightly damaged (B), seriously damaged (C), and wound-like in the body surface (D-F). Arrows indicate malformation symptoms. Bar represent 2 mm.

The percentage of individuals with at least some abnormality (HAI) (Fig. 3) and hydropsychid gill abnormality indice (HYI) (Fig. 4) were the highest in February and lowest in October. A remarked increase of HYI values with increasing concentration of pollutants concentration reflected a clear abnormality-contaminant relation, whereas the mere dicotomic classification of larvae as normal or abnormal (HAI) was less informative. High values of both HAI and HYI were associated with high concentration of contaminant. The HAI indicated deleterious effects, but failed to quantify the severity of degradation. The application of individual gill tufts, as response units in deriving HYI, revealed a simple solution to the quantification problem (Vuori, 1994).

3. Influence of physicochemical parameters on caddisfly larvae communities.

Canonical correspondence analysis is a multivariate method to calculate the relationships between biological

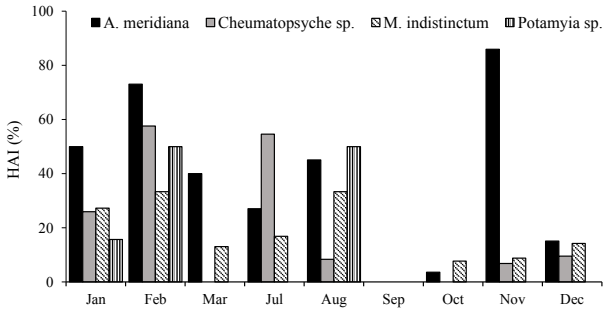


Fig. 3 Hydropsychid abnormality incidence (HAI) during January to December 2019

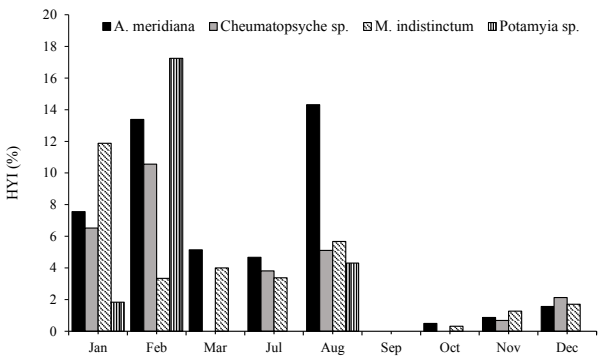


Fig. 4 Hydropsychid gill abnormality indice (HYI) during January to December 2019

assemblages of species and their environment (Ter Braak & Verdonschot, 1995). CCA revealed association between aquatic insect species and environmental variables at different months. Eigenvalue associated with each axis equal the correlation coefficient between species scores and environmental variables scores (Gauch, 1982).Thus an eigenvalue close to one will represent a high degree of correspondence between species and environmental variables, and an Eigenvalue close to zero will indicate very little correspondence (Palmer, 1993). In the present study, total eigenvalue 0.87 indicated a high degree of correspondences between species and environmental variables (Table 3).

The axis 2 was found to be strongly positive associated with ammonia-nitrogen (NH_3-N), water temperature (WT) and pH, but total dissolved solids (TDS) was found to be inversely associated with each other (Fig. 5). The species; *Amphipsyche meridiana* was positively correlated with all the measured variables, except with the TDS in February and October. Negative association of *Cheumatopsyche* spp. and *Macrostemum indistinctum*

with TDS in November and December. In the stream water, ammonia-nitrogen concentration was high ranging from 0.08±0.04 (January) to 0.72±0.04 mg/L (April). According to (PCD, 2014) ammonia-nitrogen concentrations over 0.5 mg/L will certainly have impact on a river. The local anthropogenic activities could be the discharges from intensive and prolonged agricultural activities and discharges from industrial and domestic wastes. Agricultural activities may introduce ions and metals from fertilizers and other agrochemicals (Hamid et al., 2020). The higher incidence of gill abnormalities in hydropsychid larvae may reflect the cumulative impact of contaminant exposure via food and water during larval development. As the increasing incidence of morphological abnormalities in the larvae imply an increased concentration of pollutants in the tissue (Vuori & Kukkonen, 1996). Prommi (2011) studied the altered gills morphology in *Amphipsyche meridiana* from the Pasak Jolasit Dam Outlet, central Thailand. The results showed that the tracheal gills were mostly altered followed by body surface and anal papillae. Factors such as conductivity, and inputs of orthophosphate, sulfate, turbidity, other contaminants and/or stream profiles may effect to the abnormalities, corresponding with the work by Prommi & Thamsenanupap (2013) and Ninon & Prommi (2019).

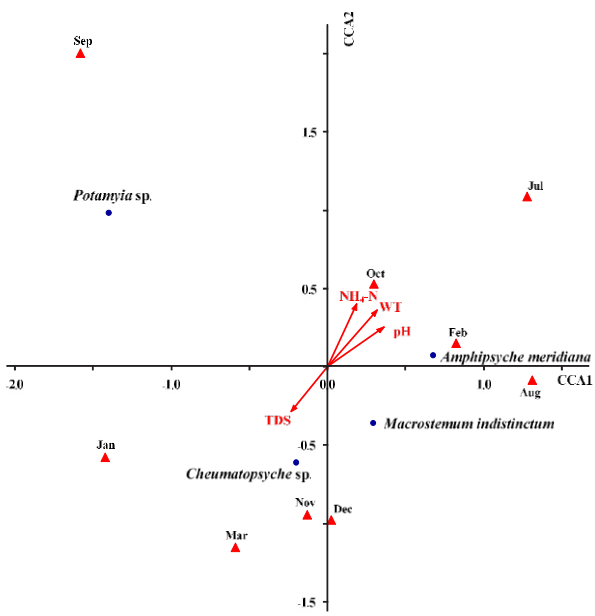


Fig. 5 CCA showing the correlation between caddisflies larvae taxa and physicochemical variables

Table 3 Summary of CCA for the caddisfly larvae and water quality variables. Axis 1 and Axis 2 significantly followed the monte-carlo permutation procedure ($P<0.05$)

	Axis 1	Axis 2	Axis 3
Eigenvalue	0.705	0.165	0.128
Variance in species data			
% of variance explained	65.2	15.3	11.8
Cumulative % explained	65.2	80.4	92.3
Pearson Correlation, species–environments	0.963	0.934	0.992
Water temperature	0.094	**0.439**	0.462
Dissolved oxygen	-0.005	0.147	0.483
pH	0.181	**0.374**	0.359
Total dissolved solids	-0.058	**-0.346**	-0.518
Ammonia-nitrogen (NH_3-N)	-0.035	**0.410**	0.509
Nitrate-nitrogen (NO_3-N)	-0.210	0.085	0.574
Orthophosphate (PO_4^{3-})	0.205	-0.190	0.525

Conclusion

The altered gill morphology in hydropsychids larvae related to multiple environmental variables. We can conclude that the malformation in tracheal gills were highly correlated with water temperature, pH, ammonia-nitrogen, and total dissolved solids. Our data indicate that the malformation in tracheal gills could be used as an early warning in aquatic ecosystems combine with physical and chemical factor.

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Effects of Exercise on Physical Performances among Frail Older Adults: A Review Study

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Abstract

Frailty is a crucial concept in determining physiological reverses among older adults. Physical frailty can predict the vulnerability for community- dwelling older adults as the functional performance declines. Recently, nurses and health care professionals have implemented the strategies to enhance physical capacity among frail older adults. Even the clinical guidelines of physical activity and exercise recommendations are widely published, older adults still engage in a low level of physical exercise. It is notable that exercise intervention is effective way to prevent frailty. This review indicates that a combination of aerobic, resistance exercise, and multicomponent exercise can improve physical performance such as increase muscle strength, balance, muscle mass, gait speed and prevent falls. Frailty is a reversible condition. Therefore, encouraging older adults to perform physical exercise is needed to maintain physical functioning as well as promote quality of life. Nurses play a significant role in frailty recovering both in hospitals and in aged care facilities.

Introduction

One of the geriatric conditions among older adults is frailty (Hoogendijk et al., 2018; Sieber, 2017). Frailty affects between a quarter and a half of people older than 70 years (Hoogendijk et al., 2018). Frailty is typically developed by the aging-related decline in strength, mobility, lean muscle mass, and activity level as well as malnutrition (Forman & Alexander, 2016; Mareschal et al., 2019). Frailty is claimed as an independent predictor for visiting urgent care in the emergency room, increased risk of falls, activities of daily living (ADL) disability, hospitalization, and mortality (Santos-Eggimann & Sirven, 2016).

Frailty is a biological syndrome of increased physiological vulnerability and resistance to stressors caused by multiple impairments in organ function and the diminishment of physiological deteriorations (Forman & Alexander, 2016). It affects three physiological systems including the immune, endocrine, and neuromuscular changes (Pillatt et al., 2019). Immunological changes include the elevation of pathobiology in the inflammatory biomarkers' interleukin-6 and C-reactive protein (Afilalo et al., 2014). These biomarkers predict twice the mortality rate compared with non-frail older adults (Afilalo et al., 2014). Endocrine changes may decrease some hormones such as testosterone and estrogen. On the other hand, the level of cortisol is induced in the circulation (Pillatt et al., 2019). Neuromuscular changes involved in the loss of muscle mass and muscular strength. These are the

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clinical manifestations of sarcopenia (Pillatt et al., 2019). Thus, frailty affects multisystem dysregulations. Typically, older adults are determined as a vulnerability group and frailty is also related to morbidity and mortality consequently, loss of dynamic homeostasis and functional decline is accelerated by frailty. Frailty is related to maladaptive response of stressor and hormone in body that presented in frail older adults with chronic diseases (WHO, 2016).

The validated tool to assess frailty in older adults is Fried Frailty Index as clinical phenotype which is widely used to categorize the frailty into 5 key domains including a decline in walking speed, weakness as measured by low grip strength, low physical activity, exhaustion by self-report or poor endurance, and unintentional weight loss at least 10 pounds, have been reported in the past year. The clinical phenotypes presented at least 3 of 5 domains indicating frail (Fried et al., 2004). Physical frailty is a major problem of older adults and frailty is related to muscle wasting, cachexia, and catabolic circulation failure (Chen et al., 2014; Dent et al., 2016). Physical frailty can affect in activity of daily life. For example, older adults had a difficulty in walking and a difficulty in maintaining balance (McPhee et al., 2016). Indeed, the functional decline is accelerated, and the body mechanism starts to fail (Forman & Alexander, 2016). Therefore, we synthesized the empirical literature across studies on exercise intervention among frail older adults, the content of the intervention, the nurse's role, and the outcomes of the intervention. The purpose of this review was to summarize the effects of exercise intervention on physical performances among frail older adults in order to clarify the state of the science and determine whether interventions are clinical enough to use as a guide in promoting physical functioning.

Search Strategy

This is a review aimed at determining the effects of exercise intervention for frail older adults. We performed a literature search up to January 2020 from databases including CINALH, PubMed, Ovid-Medline and Scopus. Search terms were (frail* AND (exercise OR physical exercise) AND physical performance*). We retrieved published articles in the English language indicating the effects of exercise intervention on physical performance. The findings across studies were synthesized through a narrative review.

Exercise intervention for frail older adults

Frailty is reversible (Seiber, 2017). Physical frailty can be treated with nutritional and pharmacological interventions (Dent et al., 2016; Mareschal et al., 2019). Several groups of researchers had dramatically explored the effect of interventions for frail older adults (Bray et al., 2016; McPhee et al., 2016; Vina et al., 2016). This includes a home-based physical exercise program, group fitness programs, adequate nutrition consultation, supervised homebased exercise, supervised computerized balance training provided individually (Bray et al., 2016). Besides, tailored management of frailty condition have been focused for inpatients, outpatients, and older adults in the community with a combination by hormone replacement, problem-solving therapy, group sessions, group as well as individual educational sessions by geriatricians for persons being at risk of frailty, a single home visit by a health professional, multiple home visits by a nurse, multiple home visits by a nurse and alert button and cognitive training (Apostolo et al., 2018).

However, one of the most prominent treatment strategies for enhancing physical capacity among frail older adults is exercise (Vina et al., 2016). Frail older adults need to perform physical activity to reserve functional capacity (Apostolo et al., 2018). Researchers have intervened those kinds of physical activity and exercise to promote functional ability. Nevertheless, there is no evidence to assure whether the type of exercise that is beneficial and practical for frail older adults (Bray et al., 2016; Liu & Fielding, 2011). Exercise intervention aimed at reversing the frailty phenotype as well as enhancing positive functional impacts on frail older adults (Apostolo et al., 2018; Bray et al., 2016). Aerobic exercise training can improve maximal oxygen intake (VO_2 peak) and increase muscle mass, while resistance exercise training focuses on increasing muscle strength and motor performance (Aguirre & Villareal, 2015; Liu & Fielding, 2011). The existing evidence supported the advantage of exercise to promote gait ability and address multiple domains of strength, endurance, and balance (Bray et al., 2016; Liu & Fielding, 2011; Pilatt et al., 2019). It is recommended that exercise intervention can be used to delay the onset of frailty and prevent functional decline (Chen et al., 2018; McPhee et al., 2016).

Exercise training intervention is often used to promote physical performances among frail older adults (Vina et al., 2016). Exercise training has been recognized

to have delayed physical dependence such as, sitting and standing, balancing, agility, and ambulation in older adults (Gine-Garriga et al., 2014; Liu & Fielding, 2011; Rodriquez-Larrad et al., 2017). The benefits of exercise among physically inactive patients have been recognized to enhance physical movement in frail older adults (Aguirre & Villareal, 2015; Gine-Garriga et al., 2014; Pillatt et al., 2019). Aerobic exercises are widely used to recover a frailty (Aguirre & Villareal, 2015). The most empirical evidences of aerobic exercise evidence supported altering the frailty phenotype, including improvement in the maximal oxygen uptake (VO_2 peak) and increased muscle mass (Bray et al., 2016). VO_2 peak refers to the maximum rate of oxygen consumption measured during vigorous exercise and to be closely related to submaximal endurance exercise capacity and exercise tolerance (Bray et al., 2016). Resistance exercise emphasizes on how well muscle strength decreases with aging. Muscle strength generally decreases about 12% to 15% per ten years, in both male and female adults respectively (Shafiee et al., 2017).

The strength of the research-based evidence supporting the exercise intervention

The evidences suggested that regular physical activity or exercise training to frail older adults are recommended in clinical settings and communities. The guidelines from the Centers for Disease Control and Prevention (CDC) stated that older adults over 65 years should participate in 300 minutes of moderate-intensity aerobic exercise per week such as brisk walking, and muscle-strengthening activities on 2 or more days a week or participate in 150 minutes of rigorous-intensity activity a week such as jogging, a heavy garden, and dancing. That is beneficial for all major muscles (legs, hips, back, abdomen, chest, shoulders, and arms) (CDC, 2020). Likewise, strong scientific evidence showed that physical activity helped to maintain physical performance in older adults.

Aerobic exercise is widely used as a physical training. Bilateral leg extension and bilateral knee extension muscles were approached for leg extensor muscles. For balance and gait retraining exercise, the semitandem foot standing, line walking, stepping practice, walking with obstacles, exercises on foam pads sequence, and changing the base of support and weight transfer from one side to another side of the legs (Cadore et al., 2013). The duration for exercise training was 40 minutes including, (1) 5 minutes for warm up (2) 10 minutes for balance and gait retraining, 20 minutes for resistance training, and 5 minutes for stretching (Cadore et al., 2013). Measuring outcomes included gait ability, TUG (Time-Up and Go) test, gait velocity test (GVT), verbal GVT and counting GVT, FICSIT-4 tests, Barthel Index (BI), and a manual dynamometer for muscle strength (Cadore et al., 2013; Pedroli et al., 2019). However, the exact amount of exercise training needed is unclear because of the various characteristics and the adverse outcomes of frailty.

Multicomponent Exercise Program (MEP) is a structured program focused on combination among resistance, balance, and flexibility exercises (de Labra et al., 2015). The characteristics of MEP include perform exercise continuously with a combination a single daily activity, enhance endurance and balance training, and increase volume, intensity, and tolerance (WHO, 2016).

MEP usually includes gait, coordination, and physical function training (CDC, 2020). MEP typically can perform at homes or in communities which serve as community-based activities such as dancing, yoga, tai chi, and/or sport (CDC, 2020). Older adults may consider MEP as they incorporate multiple types of physical activity in their daily living (Giné-Garriga et al., 2014). Regarding exercise intervention for frail older adults, many studies focus on improving the overall health status of frail older adults regarding MEP. For example, Cadore et al. (2013) studied a MEP for enhancing the overall health status of frail older adult individuals. MEP is a combined program of endurance, strength, coordination, balance, and flexibility exercise (Giné-Garriga et al., 2014). MEP was performed on a 12-week (twice weekly) of randomized control trial (RCT) using MEP among 24 community-dwelling older adults with a mean age of 91.9 years. MEP composed of upper and lower body resistance to increase muscle power loads combined with balance and gait retraining exercise (Cadore et al., 2013). Frail older adults performed resistance exercise by approaching their major upper and lower limb muscles. For upper limbs, the seated bench press was performed (Rodriguez-Larrad et al., 2017). The finding showed that MEP decreased time spent on performing TUG.

MEP could enhance the strength and muscle power among older adults (de Labra et al., 2015; Pillatt et al., 2019). Engaging in MEP reduces the risk of frailty both communities (de Labra et al., 2015) and institutional cares (Rodriguez-Larrad et al., 2017). MEP is also feasible in institutional care setting.

Rodriguez-Larrad et al. (2017) studied the effects of MEP on physical functioning among frail older adults in nursing home. In this study, the MEP program consisted of twice a week within 48 hours totally for 6-month period intervention regarding strength, balance, and walking training within 45 minutes. Firstly, the intervention provides 5-minute brief warmup focusing on range of motion exercises and mobility of joints and extremities. Next, a 25-min of strength training aims to enhance individuals' upper and lower body functional capacity. Balance training is a third step to promote gait and balance. The activities include 10-minute totally for standing on tips and heels, one legged stand, semi-tandem/tandem exercise, circuit training, stepping, and ball reaching. Lastly, a 5-minute of cooling down is focused on stretching, breathing, and relaxing exercise.

The goal of the first 3 months was to increase muscle strength while the 3-month later aimed to improve functional capacity. The finding found that the MEP could improve functional performances, sedentary behavior, cognition and emotional status as well as biological marker such as interleukin-6 that relates to frailty and physical performance.

To sum up, older adults with frailty need MEP because frail older adults need aerobic exercise to preserve physiological function and humoral balance while resistance exercise also can improve activity daily living. MEP is considered to be the most effective strategy for improving gait, balance and strength, preventing fall incidence, and promoting the functional capacity among frail older adults.

Physical performance outcomes from exercising

Based on the review of the studies, exercise training intervention provided positive outcomes on physical performances as following.

1. Gait and Balance

TUG measures gait and balance regarding the time needed to complete a series of functional tasks including standing up from a chair, walking 3 meters, turning around, returning to the chair, and sitting down on a chair (Cadore et al., 2013). The finding showed that MEP decreased time to spend for performing TUG ($p < .05$) (Cadore et al., 2013)

2. Walking

Gait speed refers to a test that requires frail older adults to walk a certain distance. Gait speed is one of the predictors for survival in older adults (Giné-Garriga et al., 2014). A systematic review and meta-analysis of Giné-Garriga et al. (2014) showed that exercise increased gait speed and frail older adults who received exercise training walked faster than the control group. Liu and Fielding (2011) suggested that aerobic exercise such as walking is accessible for frail older adults to engage and maintain their daily activity and physical performance.

3. Fall

Fall is one of the most important health outcomes. Fall is measured by using a questionnaire for asking older adults on the history of falls and incidence in previous 12 months. A review study showed that exercise could reduce the risk of falls by 57% among elder women with frailty after undergoing a resistance training program for 25 weeks compared with from baseline (Liu-Ambrose et al., 2004). The empirical finding is supported by another study. Cadore et al. (2013) found that the MEP decreased fall incidence at post-training compared with pre-training ($p < .01$). Muscle strength. Muscle strength is usually measured by using a manual dynamometer. Cadore et al. (2013) found that hip flexion and knee extension strength were increased after exercise intervention among exercise intervention group ($p < .01$ and $p < .05$ respectively)

In summary, the strengths of MEP comprised of resistance training, aerobic exercise, and balance training. Upon the review of exercise training, the positive outcomes of physical performance were affected by gait speed, TUG, fall, and muscle strength. As frailty is reversible, frail older adults would perform a better ADL such as walking with longer distances, spending less time to do TUG, preventing falls, and gaining muscle strength.

Gaps in the knowledge base related to the intervention

Upon the previous intervention studies, it is known that both aerobic, resistance exercise intervention and multicomponent exercise can improve physical performance, both increased muscle strength and muscle mass. Frail older adults in community have faced with physical performance assessment regarding the multicomponent exercise. Moreover, the rate of exercise progression and intensity remain unknown for frail older adults. In addition, the researchers have not emphasized specific diseases that older adults might have different experiences on frailty, such as different diseases. The researchers did not closely monitor the frail older adults and the progress of the exercise. A further

study was suggested to observe the levels of frailty during program intervention (Bray et al., 2016). The gap of study needs to be addressed to improve physical performance in frail older adults by tailored multicomponent exercise intervention and concern monitoring on the ability to perform physical exercise individually regarding functional abilities measure. The further step should examine the effects of multicomponent exercises in frail older adults with chronic diseases to enhance physical performance.

The challenges that limit the use of the intervention

Frailty is commonly found in older people with advanced age. The effect on the phenotype of frailty needs to be concerned. Although the studies on multi-component exercises are beneficial for frail older adults, the effects of exercise on the various characteristics of frailty and the adverse outcomes of frailty are limited in using the intervention. This involves differences in a specific time, frequency, intensity, measuring, and setting of the intervention, and the level of health status in frail older adults. The adverse outcomes might occur if the older adults have musculoskeletal complaints or the level of frailty, particularly in exercise training in female older adults. Therefore, the effectiveness of intervention should emphasize these issues for randomizing the subjects. In addition, the measures for a screening of frailty in older adults need to be concerned about investigating the tolerance of frailty, the duration cannot take too long in a longitudinally study. The nutritional status might be related to the level of frailty as a study has shown that the protein-calories and vitamin D supplementation are related to increasing muscle mass.

Nursing intervention for frail older adults

Frail older adults are at risk for adverse effects on their health. They also considered as the most significant consumers of health resources across both acute and long-term care (Kojima et al., 2019). Nurses play a significant role in caring for frail older adults in terms of assessment, identifying, and managing older adults, who are susceptible to or experiencing frailty (Maxwell & Wang, 2017). Early stages of frailty are commonly found in older adults who live in the community, while they have the high risk of frailty (Chen et al., 2018). Identifying frailty among older adults in the early stage can prevent them from functional dependence and improve or maintain functional independence (Maxwell & Wang, 2017). Taking care of frail older adults is complicated because they have an increased burden of symptoms that cause frailty, thus increasing external help (Kojima et al., 2019). The aims of nursing care of frail older adults are maintaining a state of homeostasis. Therefore, multiple interventions are necessary for this group. The interventions for frail older adults include 1) exercise including resistance, strength, physical exercise, and lingual exercise, 2) adequate nutritional maintenance, 3) apt environmental modifications, and 4) information on health (Maxwell & Wang, 2017).

Conclusion

Frail older adults are at risk for adverse effects on their health. The literature found that physical exercise was a challenge to perform. Exercise intervention for frail older adults in promoting their physical performances is crucial. Nevertheless, frail older adults need to adhere with physical exercise continuously at least 6 months in order to promote health-enhancing physical exercise. However, we should emphasize the nutritional maintenance and health literacy through frail older adults. Nursing interventions for frail older adults include physical exercise, adequate nutritional maintenance, appropriate environmental modifications, and information on health; which should be provided continually until stable.

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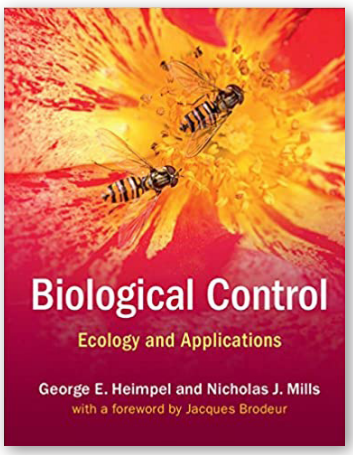
Book Review

Nathapong Matintarangson



Book name:	Biological Control Ecology Applications
Series:	Entomology
Author:	Heimpel, G.E., & Mills, N.J.
Published:	Cambridge University Press, 2017
Paperback:	368 pages
Language:	English
ISBN:	978-0-52184514-4

Biological control is carefully placed within the context of the ecological principles of biological invasions. The authors argue that we have now reached a point where the balance between risks and benefits of biological control is no longer contested. This acceptance brings with it new concepts and approaches to environmental risk assessment. There are new tools which can enhance and better measure biological controls and with them there emerges a focus on conservation and natural biological control and the growing interest in wider applications of biological control, all of which are lucidly.

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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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.

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