การคัดกรองฤทธิ์ต้านเอนไซม์ไลเปสจากพริกพื้นบ้านไทย

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บทคัดย่อ

โรคอ้วนเป็นปัจจัยความเสี่ยงหลักของการเกิดโรคไม่ติดต่อเรื้อรัง ซึ่งการรักษาโรคอ้วนเกี่ยวข้องกับการย่อยไขมันผ่านการยับยั้งเอนไซม์ไลเปส วัตถุประสงค์ของการศึกษานี้คือ เพื่อทดสอบฤทธิ์ต้านการทำงานของเอนไซม์ไลเปสจากพริกพื้นบ้านไทย (สกุล *C. annuum* และ *C. frutescens*) ตัวอย่างพริกถูกสกัดด้วยสารละลายเอทานอลที่ความเข้มข้นร้อยละ 70 โดยปริมาตร ที่อุณหภูมิ 60°ซ ผลการศึกษาพบว่าพริกกะเหรี่ยงสีเขียว (สกุล *C. frutescens*) มีฤทธิ์ต้านเอนไซม์ไลเปสสูงสุด (ร้อยละ 49) ในขณะที่พริกกะเหรี่ยงสีแดงมีฤทธิ์ต้านเอนไซม์ร้อยละ 36 ในทางตรงกันข้าม พริกหยวกสีแดง (สกุล *C. annuum*) มีฤทธิ์ต้านเอนไซม์ไลเปสต่ำสุด (ร้อยละ 8) ในขณะที่พริกหยวกสีเขียวมีค่าต้านร้อยละ 13 โดยพริกสีเขียวส่วนใหญ่มีแนวโน้มการยับยั้งมากกว่าพริกสีแดง ซึ่งอาจมีสาเหตุมาจากปริมาณสารสำคัญที่อยู่ในพริกแต่ละชนิดมีความแตกต่างกัน โดยพริกกะเหรี่ยงสีเขียวที่มีฤทธิ์ต้านเอนไซม์ไลเปสสูงที่สุดถูกพบว่า มีปริมาณกรดเฟอรูลิก กรดพารา-คูมาริก และกรดซิแนฟปิก สูงกว่าพริกกะเหรี่ยงสีแดง นอกจากนี้ พริกในสกุล *C. frutescens* มีแนวโน้มยับยั้งเอนไซม์ไลเปสได้ดีกว่าพริกในสกุล *C. annuum* ดังนั้น ปัจจัยภายใน เช่น ระยะสุก และสกุลของพริกอาจส่งผลต่อฤทธิ์ต้านเอนไซม์ไลเปสได้

คำสำคัญ: พริก การยับยั้งเอนไซม์ไลเปส โรคอ้วน

***ผู้รับผิดชอบบทความ**

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The Screening of Anti-lipase Activity in Thai Local Chili Peppers

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Abstract

Obesity is a major risk factor of chronic diseases, leading to deaths of more than 10% of the global population. Medicinal treatment of obesity involves control of lipid degradation *via* inhibition of the key enzyme, lipase, which hydrolyzes triglyceride into glycerol and free fatty acids. Thus, the purpose of this study was to reveal lipase inhibitory activities of Thai local chili peppers (*C. annuum* and *C. frutescens*). All chili peppers were extracted by 70% (v/v) ethanol in a 60 °C water bath shaker. The anti-lipase activities were determined by colorimetric coupled assay using a 96-well microplate reader. Among all investigated chili peppers, the immature green bird chili (*C. frutescens*) was found to exhibit the highest anti-lipase activity (49% inhibition), while its mature red counterpart provided 36% inhibition. On the other hand, the mature red banana pepper (*C. annuum*) exhibited the lowest inhibition (8%), while its immature green pepper exhibited 13% inhibition. Overall, most immature green chili peppers tended to exhibit higher percentage of inhibition than their mature red counterparts, which might be due to different types of quantity of bioactive compounds found in particular chili peppers. Green bird chili with the highest lipase inhibitory activity was found to exhibit the higher content of ferulic acid, *p*-coumaric acid and sinapic acid than the red one. Besides, chili peppers in *C. frutescens* species tended to exhibit higher anti-lipase activities than the ones in *C. annuum* species. Therefore, the internal factors such as maturity and species may strongly influent anti-lipase reaction.

Keyword: Chili pepper, Lipase inhibition, Obesity

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Introduction

Obesity is a major risk factor of chronic diseases such as diabetes, cardiovascular disease and cancer[1]. Characteristics of obesity are due to imbalance between energy intake and energy expenditure, in which the excessive energy accumulation is deposited in the body as adipose tissue. Medicinal treatment of obesity involves control of lipid degradation *via* inhibition of the key enzyme, lipase. This enzyme hydrolyzes triglyceride into glycerol and free fatty acids, which subsequently stored in adipose tissue. Over-consumption of oily/fatty foods could lead to obesity. The treatment of obesity consists of reducing energy intake through gastrointestinal tract, improving digestion and inhibiting fat absorption[2]. Currently, two functional obesity-treating drugs including[1] an anorectic/appetite suppressant that acts on the central nervous system (sibutramine) and lipase inhibitor (orlistat) are commercially available[3]. However, adverse effects of sibutramine consist of dry mouth, constipation and insomnia. Therefore, it is approved for short-term (around 3 months) therapy by Food and Drug Administration (FDA)[3, 4]. Safer anti-lipase agents are, thus, commonly used for treating obesity through blocking or reducing energy intake from fat dietary[5]. Orlistat is an anti-obesity drug and a competitive lipase inhibitor. The half maximal inhibitory concentration (IC_{50}) of orlistat is 0.15 μM by a measurement of porcine pancreatic lipase inhibitory activity *in vitro* using *p*-nitrophenyl butyrate as a substrate[6]. Orlistat, however, has several side effects, including depressive symptom, increased blood pressure, increased defecation, fatty oils evacuation, oily spotting and decreased endogenous vitamin level[7, 8].

Interestingly, some natural products from bacteria, fungi, marine products and medicinal plants had been reported to possess similar biological property against lipase with no/less side effect. Some of the bioactive compounds extracted from these sources were previously reported as anti-lipase agents such as chitosanoligosaccharides, polyphenols, saponin, triterpene, terpenoid, phytosterol and alkaloid[9-11]. Polyphenols possess effective approach for obesity prevention, which related to inhibition of enzymes involved in lipid metabolism, including pancreatic lipase, lipoprotein lipase and glycerophosphate dehydrogenase[12].

Chili pepper had previously shown the ability to fight against obesity. Korean fermented red pepper paste was reported to reduce body fat gain, lipid levels of adipose tissue, and lipid accumulation in 3T3-L1 adipocytes[13]. It was also found that red pepper could decrease appetite and

subsequent protein and fat intakes in Japanese females and energy intake in Caucasian males[14]. The major alkaloid found in Capsicum genus or chili pepper is capsaicinoids, especially capsaicin, followed by dihydrocapsaicin[15]. Capsaicin could induce apoptosis and inhibit lipid accumulation in 3T3-L1 preadipocytes and adipocytes, indicating a cell population growth of 3T3-L1 preadipocytes assessed with MTT assay[16]. Moreover, capsaicin attenuates obesity-induced inflammation and obesity related metabolic disorders[17]. In addition, chili peppers contain flavonoids and phenolic acids with a powerful anti-lipase function such as myricetin, kaempferol, luteolin, quercetin and apigenin[18, 19]. Even though there is no previous report on anti-lipase properties of Thai local chili peppers, it is most likely that these chili peppers possess the ability to fight against lipase reaction as well. Therefore, the objective of this study was to investigate the effects of species and maturity stage of Thai local chili peppers on lipase reaction and determine the content and types of flavonoids and phenolic acids of selected Thai local chili peppers with high lipase inhibitory activities.

Material and Methods

1. Chemicals

All chemicals and reagents including, 5–5'–dithiobis(2–nitrobenzoic acid) (DTNB), N–phenacyl–4,5–dimethylthiazolium bromide (DMPTB), Triton X–100, potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), were received from Sigma–Aldrich (St. Louis, MO, USA).

Standards for analysis of bioactive compounds including myricetin, luteolin, isorhamnetin, quercetin, hesperitin, kamepferol, apigenin, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, galic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, *t*-cinnamic acid, naringenin and ferulic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Solvents including ethanol (EtOH), acetronitrile (ACN), and methanol (MeOH) were received from RCI Labscan (Bangkok, Thailand). Tertiarybutyl-hydroquinone (tBHQ) and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and hydrochloric acid (HCl) were received from Merck (New Jersey, USA).

Enzymes and proteins used in this study including *Candida rugosa* lipase (Type 8, ≥700 unit/mg) and bovine serum albumin (BSA, ≥98% agarose gel electrophoresis) were received from Sigma–Aldrich (St. Louis, MO, USA).

2. Sample collection and preparation

Thai local chili peppers (23 samples) in *C. annuum* and *C. frutescens*

specie were collected from Kanchanaburi province, Thailand during November (2012) and August to October (2013) due to suitable climate for harvesting chili fruits (Table 1). The weights and sizes of chili peppers were measured within a same day of harvesting to avoid weight loss and fruit body shrunken from dehydration. After that, all chili peppers were cleaned, chopped and freeze-dried before being ground into fine powder by a grinder and stored at -20°C. Each sample (0.2 g dry weight) was then resuspended in 70% (v/v) ethanol (8 mL) and vortexed for 5 min. The mixture was sonicated for 10 min and continued shaking at 100 strokes per minute in a temperature-controlled water bath at 60°C for an hour. The sample was then centrifuged at 1190*xg* for another 10 min at 4°C. The supernatant was collected and stored at -20°C for further analysis.

3. Lipase inhibitory assay

The lipase inhibitory activity was determined by a colorimetric microplate assay. The enzyme reaction consisted of 100 µL *Candida rugosa* lipase (1 µg well^{-1}) in enzyme buffer (50 mM Tris, 10 mM KCl, 1 mM EDTA containing 0.1% (w/v) BSA, pH 8.0), 40 µL 2,3-dimercapto-1-propanol tributyrate (DMPTB, 50 µM well^{-1}) in assay buffer (50 mM Tris containing 10 mM KCl and 1 mM EDTA, pH 7.2), 10 µL 5,5'-dithiobis (2-nitro benzoic acid) (DTNB, 0.8 mM well^{-1}) in 50 mM KPB (pH 7.4) and 50 µL chili pepper extracts (5 mg/mL well^{-1}). The enzyme reaction was monitored as a colorless DMPTB (a substrate) being hydrolyzed by the enzyme to 2,3-dimercapto-1-propanol. This product subsequently interacted with DTNB (an indicator) to form yellow 2-nitro-5-mercaptobenzoic acid, which was kinetically monitored at 412 nm for an hour using a 96-well microplate reader (BioTek Instruments, Inc., Winooski, VT) with a Gen5 data analysis software. The percentage of lipase inhibition was calculated by the equation;

$$\% \text{ inhibition} = 100 \times \left[1 - \left(\frac{(B-b)}{(A-a)}\right)\right],$$

where A is initial velocity of control reaction with lipase, a is an initial velocity of control reaction without lipase, B is initial velocity of the enzyme reaction with extract and b is an initial velocity of the reaction with extract but without enzyme. The inhibitory activity was expressed as mean ± standard deviation (SD). The significance of difference was expressed as different lipase inhibitory activities in all chili peppers at $p<0.05$ using one way analysis of variance (ANOVA) and Turkey's multiple comparison tests. Meanwhile, the significance of flavonoids and phenolic acids were compared with standard (orlistat) using independent samples T-test at $p<0.01$. All statistical analyses were performed using IBM SPSS

Statistics version 19.0 (IBM Corp, Armonk, NY).

The enzyme kinetics were performed in the 96–well plate utilizing 100 µL *Candida rugosa* lipase (1 µg well^{-1}) in enzyme buffer (50 mM Tris, 10 mM KC l, 1 mM EDTA containing 0.1% (w/v) BSA, pH 8.0), 40 µL DMPTB (15-25 µM well^{-1}) in assay buffer (50 mM Tris containing 10 mM KCl and 1 mM EDTA, pH 7.2), 10 µL DTNB (0.8 mM well^{-1}) in 50 mM KPB (pH 7.4) and selected bioactive compounds in 50% v/v DMSO. The initial rate data were fitted to a Michaelis-Menten equation with least squares fit parameters using GraphPad Prism software version 6.01 for Windows (GraphPad Software, Inc., San Diego, CA, www.graphpad.com). Inhibition types and kinetic parameters including the inhibitory constant (K_i), the Michaelis constant (K_m), the maximal velocity (V_{max}) were evaluated by Lineweaver-Burk plot and secondary Lineweaver-Burk plot. The specific activity of initial velocity was calculated by the equation;

$$V=\frac{(B-b)}{\varepsilon_\lambda * L} * \frac{\text{total volume}}{\text{weight enzyme}},$$

where V is specific activity (µmol/min/mg/), B is initial velocity of the enzyme reaction with inhibitor (min^{-1}), b is an initial velocity of the reaction with inhibitor but without enzyme (min^{-1}), ε_λ is molar extinction coefficient (cm^{-1}M^{-1}), and L is path length (cm). Orlistat (3-7 µM), a commercially available lipase inhibitor, is used as a control.

4. Identification of flavonoids and phenolic acids

Identification of flavonoids and phenolic acids were determined by HPLC analysis. Sample extraction for identification of flavonoids and phenolic acids was performed according to previous procedure with some changes as follows[20]. Freeze-dried sample was hydrolyzed with 62.5% (v/v) MeOH (40 mL) containing 0.5 g/L tBHQ and 6 N HCl (10 mL) in a temperature-controlled water bath shaking at 80°C for 2 hours. The hydrolyzed sample was cooled in ice bath for 5 min before neutralizing with 1% (v/v) ascorbic acid (100 µL). The extract was then sonicated for 5 min and was adjusted the volume to 50 mL with 62.5% (v/v) MeOH. The extract was filtered using 0.22 µm PTFE syringe filter before loading to the Zorbax Eclipase XDB-C18 column (4.6 mm x 150 mm, 5 µm) (Agilent Technologies, Santa Clara, CA, USA). The HPLC system was consisted of a series of gradient mobile phases, including Milli-Q water containing 0.05% (w/w) TFA (solvent A), MeOH containing 0.05% (w/w) TFA (solvent B), and acetonitrile containing 0.05% (w/w) TFA (solvent C). The constant flow rate was set at 0.6 mL/min. The bioactive compounds in the sample were monitored at 338 nm and identified by comparing the

retention times (R_t) with those of the standard sets of flavonoids (quercetin, kamepferol, isorhamnetin, myricetin, apigenin, luteolin, naringenin and hesperitin) and phenolic acids (gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, syringic acid, vanillic acid, caffeic acid, *p*-courmaric acid, ferulic acid, sinapic acid and *t*-cinnamic acid).

Results and Discussion

The lipase inhibitory activity of Thai local chili peppers (5.0 mg/mL) extracted with 70% (v/v) ethanol showed that all Thai local chili peppers could inhibit lipase reaction with different degrees of inhibition (Table 1). Ethanolic extracts of Thai local chili peppers possessed anti-lipase activities within the range of 8.42-48.51% inhibition. These chili peppers can be divided according to lipase inhibitory activities, including high (>40% inhibition), middle (20-40% inhibition) and mild (<20% inhibition) inhibitory groups. Among high inhibitory group, green bird chili exhibited the highest anti-lipase activity (48.51% inhibition), followed by red spur pepper (47.67%), green hang cluster cayenne pepper (44.96%), yellowish orange spur pepper (44.45%), yellowish green spur pepper (43.24%), green cluster cayenne pepper plus spur pepper (41.88%), red big cayenne pepper (41.21%) and green round spur pepper (40.86%), respectively. Middle inhibitory group was included green medium cayenne pepper (39.43%), green big cayenne pepper (38.00%), red bird chili (36.18%), green cluster cayenne pepper (35.52%), red cluster cayenne pepper plus spur pepper (35.12%), red round spur pepper (32.04%), green yellow pepper (31.64%), red yellow pepper (29.83%), red cluster spur pepper (25.94%), red medium cayenne pepper (24.83%) and green cluster spur pepper (23.07%), respectively. In the group with mild inhibitory activities, red hang cluster cayenne pepper exhibited the highest anti-lipase activity (19.65%), followed by red cluster cayenne pepper (15.14%) and green banana pepper (12.50%), respectively, while red banana pepper exhibited the lowest activity (8.42%). Comparing to orlistat (74.82% inhibition at 6 µg/mL), it was found that these chili extracts exhibited much lower lipase inhibitory activity.

Table 1 Thai local chili peppers used in this experiment and lipase inhibitory activities.

Group	Name	Fruit Shape	Length (cm.)	Width (cm.)	Weight (g.)	Pedicel Length (cm.)	Wall Thickness (mm.)	Color	% Inhibition of Lipase*
High inhibition	Green bird chili	Elongate	3.51	0.78	1.00	3.73	0.63	Green	48.51 ± 2.12^{a}
	Red spur pepper	Elongate	12.60	1.97	15.67	4.60	2.11	Red	47.67 ± 0.67^{ab}
	Green hang cluster cayenne pepper	Elongate	8.37	0.70	2.33	2.55	0.70	Green	44.96 ± 4.09^{abc}
	Yellowish orange spur pepper	Elongate	12.60	1.97	15.67	4.60	2.11	Yellow/ Orange	44.45 ± 3.06^{abcd}
	Yellowish green spur pepper	Elongate	11.20	1.93	15.54	5.88	1.98	Yellow/ Green	43.24 ± 1.49^{abcd}
	Green cluster cayenne pepper plus spur pepper	Elongate	7.17	0.65	2.00	2.97	0.41	Green	41.88 ± 4.28^{bcde}
	Red big cayenne pepper	Elongate	6.27	0.88	2.00	3.88	1.13	Red	41.21 ± 0.29^{bcdef}
	Green round spur pepper	Round	4.03	4.50	33.25	2.79	5.39	Green	40.86 ± 1.54^{cdef}
Middle inhibition	Green medium cayenne pepper	Elongate	5.96	0.99	3.00	4.51	1.14	Green	39.43 ± 2.09^{cdef}
	Green big cayenne pepper	Elongate	6.27	0.88	2.00	3.88	1.13	Green/ Brown	38.00 ± 2.74^{defg}
	Red bird chili	Elongate	3.51	0.78	1.00	3.73	0.63	Red	36.18 ± 1.32^{efgh}
	Green cluster cayenne pepper	Elongate	6.07	0.92	3.00	6.27	1.09	Green	35.52 ± 1.67^{efgh}
	Red cluster cayenne pepper plus spur pepper	Elongate	7.17	0.65	2.00	2.97	0.41	Red	35.12 ± 0.21^{fgh}
	Red round spur pepper	Round	4.03	4.50	33.25	2.79	5.39	Red	32.04 ± 1.89^{ghi}
	Green yellow pepper	Elongate	9.63	1.77	9.33	3.83	1.47	Green	31.64 ± 1.51^{ghi}
	Red yellow pepper	Elongate	9.63	1.77	9.33	3.83	1.47	Red	29.83 ± 2.87^{hij}
	Red cluster spur pepper	Elongate	7.81	0.74	2.67	2.78	0.67	Red	25.94 ± 1.42^{ijk}
	Red medium cayenne pepper	Elongate	5.96	0.99	3.00	4.51	1.14	Red	24.83 ± 1.05^{jk}
	Green cluster spur pepper	Elongate	7.81	0.74	2.67	2.78	0.67	Green	23.07 ± 1.39^{k}
Mild inhibition	Red hang cluster cayenne pepper	Elongate	8.37	0.70	2.33	2.55	0.70	Red	19.65 ± 1.43^{kl}
	Red cluster cayenne pepper	Elongate	6.07	0.92	3.00	6.27	1.09	Red	15.14 ± 0.46^{lm}
	Green banana pepper	Triangular	11.26	3.27	37.67	2.60	1.90	Green	12.50 ± 0.72^{mn}
	Red banana pepper	Triangular	11.26	3.27	37.67	2.60	1.90	Red	8.42 ± 0.97^{n}

The percentages of lipase inhibitory activity were expressed as mean ± standard deviation (SD) of triplicates assays. The significant different letters (a-n) were expressed as differences in lipase inhibitory activities of 23 chili peppers at p value < 0.05 using one-way analysis of variance (ANOVA) test with Turkey's multiple comparison test (n=3). For example, the samples with significant letter of "a" comprise of red banana pepper, red spur pepper, green hang cluster cayenne pepper, yellowish orange spur pepper and yellowish green spur pepper. The samples with significant letter of "b" comprise of red spur pepper, green hang cluster cayenne pepper, yellowish orange spur pepper, yellowish green spur pepper, green cluster cayenne pepper plus spur pepper and red big cayenne pepper. Other significant letters were also applied for the samples in the similar meaning. *Final concentration of chili pepper extracts was 5.0 mg/mL. Numbers 1-21 are *C. annuum*, while numbers 22-23 are *C. frutescens*.

Overall, most green chili peppers tended to exhibit higher percentage of inhibition than those of the red mature chili peppers. For example, immature green hang cluster cayenne pepper was in the high inhibitory group (45% inhibition), while its mature red counterpart was in the mild inhibitory group (20% inhibition). Even though cluster spur pepper, spur pepper and big cayenne pepper in their maturity tended to exhibit higher lipase inhibitory activities than their immature counterparts, the differences in anti-lipase activities of these chili peppers were within 5% inhibition and were not significantly difference at p-value < 0.05. Besides, the highest lipase inhibitory activity belongs to green bird chili in *C. frutescens* species, while the lowest belongs to red banana pepper in *C. annuum* species. Both chili peppers in *C. frutescens* species were classified to high and middle inhibitory activity groups. Therefore, it can be concluded that internal factors such as maturity and species may strongly influent anti-lipase reaction. On the other hand, external factors including fruit lengths, fruit widths, fruit pedicel lengths, fruit wall thicknesses, fruit weights and fruit color may have little effect on lipase inhibition.

The bioactive compounds including flavonoids and phenolic acids in selected Thai local chili pepper, green bird chili, with the highest inhibitory activity were identified in attempt to explain to their potential inhibition toward key enzyme related to obesity (Table 2). Phenolic acids including *p*-coumaric acid (51.14 µg/g freeze dry (FD) weight), ferulic acid (171.15 µg/g FD weight) and sinapic acids (45.53 µg/g FD weight) (from standards including chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, gallic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, *t*-cinnamic acid, naringenin and ferulic acid) were detected in green bird chili. However, the contents of these phenolic acids in green bird chili were higher than the ones in red bird chili (*p*-coumaric acid of 32.80 µg/g FD weight, ferulic acid of 67.37µg/g FD weight, and sinapic acids of 38.70 µg/g FD weight).

Table 2 The flavonoids and phenolic acids of selected Thai local chili peppers.

Species	Chili peppers	Phenolic acids (µg/g freeze dry weight)		
		p-Coumaric acid	Ferulic acid	Sinapic acid
C. frutescens	Green bird chili	51.14	171.15	45.53
	Red bird chili	32.80	67.37	38.70

All analytical data were mean values of independent sample.

It was found that internal factors such as maturity and species strongly influent anti-lipase reaction. Most immature green chili peppers tended to exhibit higher percentage of lipase inhibition than those of the mature red chili peppers. Our experiments were corresponded to the previous reports on lipase inhibitory activities of sweet pepper (*C. annuum*), in which immature green sweet pepper exhibited higher anti-lipase activity than mature red sweet pepper[21]. Higher anti-lipase activity in immature green chili pepper than in mature red chili peppers might be corresponded to types and contents of phenolic acids. To confirm this hypothesis, the IC_{50} values of *p*-coumaric acid, ferulic acid and sinapic acids were analyzed using a plot of lipase inhibitory percentage and the logarithm of concentration of the corresponding bioactive compounds. It was found that *p*-coumaric acid, sinapic acid and ferulic acid exhibited the IC_{50} values of 6.66, 7.06 and 7.13 mM, respectively (Figure 1, Table 3). However, comparing to orlistat with the IC_{50} value 0.0034 mM, these phenolic acids are less effective inhibitors.

Table 3 Kinetic parameters of lipase inhibitory activities with selected bioactive compounds and standard inhibitor.

Bioactive compounds	IC_{50} (mM)	*Ki* (mM)	Inhibition type
Phenolic acids			
p-Coumaric acid	6.66 ± 0.41*	13.90	Mixed-type non-competitive
Ferulic acid	7.13 ± 0.58*	11.82	Competitive
Sinapic acid	7.06 ± 0.13*	8.02	Mixed-type non-competitive
Standard inhibitor			
Orlistat	0.0034 ± 0.00017	0.0022	Competitive

The IC_{50} data were expressed as mean ± standard deviation (SD) using independent-samples T-test (n=3) and * $p < 0.01$ comparing with standard inhibitor. Bioactive compounds and standard inhibitor were dissolved in 50% (v/v) DMSO.

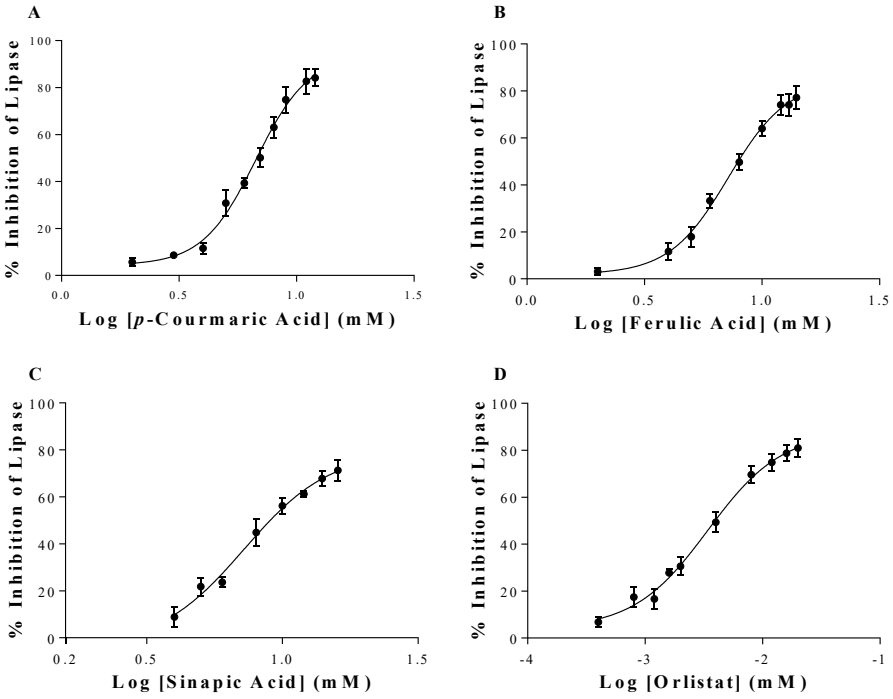

Figure 1 The plots of percentage of lipase inhibition versus log[inhibitor] of (**A**) *p*-coumaric acid, (**B**) ferulic acid, (**C**) sinapic acid and (**D**) orlistat that used to determine the IC_{50} values. Each point was mean ± standard deviation (SD) of the triplicate assays.

Meanwhile, K_i and inhibition mode of inhibitor were determined by the plot of lipase kinetics with phenolic acids. Interpreted from the Michaelis-Menten plots, the Lineweaver-Burk plots and the secondary Lineweaver-Burk plots were employed to determine mode of inhibition and K_i, respectively. The Lineweaver-Burk plots of ferulic acid and orlistat showed the intercept at the Y-axis (1/specific activity), suggesting that these two compounds are competitive inhibitors (Table 3). Besides, it was found that *p*-coumaric acid and sinapic acid were mixed-type non-competitive inhibitors with the intercepts between the Y-axis and X-axis (Table 3). Based on enzyme kinetics analysis, orlistat and ferulic acid that act as competitive inhibitors can directly interact with free enzyme to form an inactive enzyme-inhibitor complex. This formation can induce enzyme conformational change, preventing the interaction between enzyme and substrate to form enzyme-substrate complex. The inhibition mode of orlistat is corresponded with previous studies that revealed to be a competitive inhibitor[6,22]. Being a competitive inhibitor, orlistat interacts with free lipase at the enzyme active site though the covalent bonding with catalytic Ser152, preventing the substrate to interact with the enzyme. This

type of inhibitor is very efficient, thus only small amount of inhibitor (low K_i value) can effectively inhibit the enzyme. Interaction of competitive inhibitor of ferulic acid may interact with free lipase at the active site similarly to that of orlistat. However, without the ester moiety in its structure, the interaction of ferulic acid to the enzyme may not be as stable as orlistat, resulting in observable higher K_i. Furthermore, *p*-coumaric acid and sinapic acid acted as mixed-type non-competitive inhibitors. Mixed-type inhibition is the part of non-competitive inhibitor, which interacts with both free enzyme and enzyme-substrate complex. Sinapic acid was a mixed-type inhibitor with mode of action similar to non-competitive type. Thus, sinapic acid can interact with both free enzyme and enzyme-substrate complex with almost the same rate. On the other hand, *p*-coumaric acid more likely interacts with enzyme-substrate complex than with free enzyme as being indicated by the increment of X-intercept when increase concentration of inhibitor in the secondary Lineweaver-Burk plots (Figure 2).

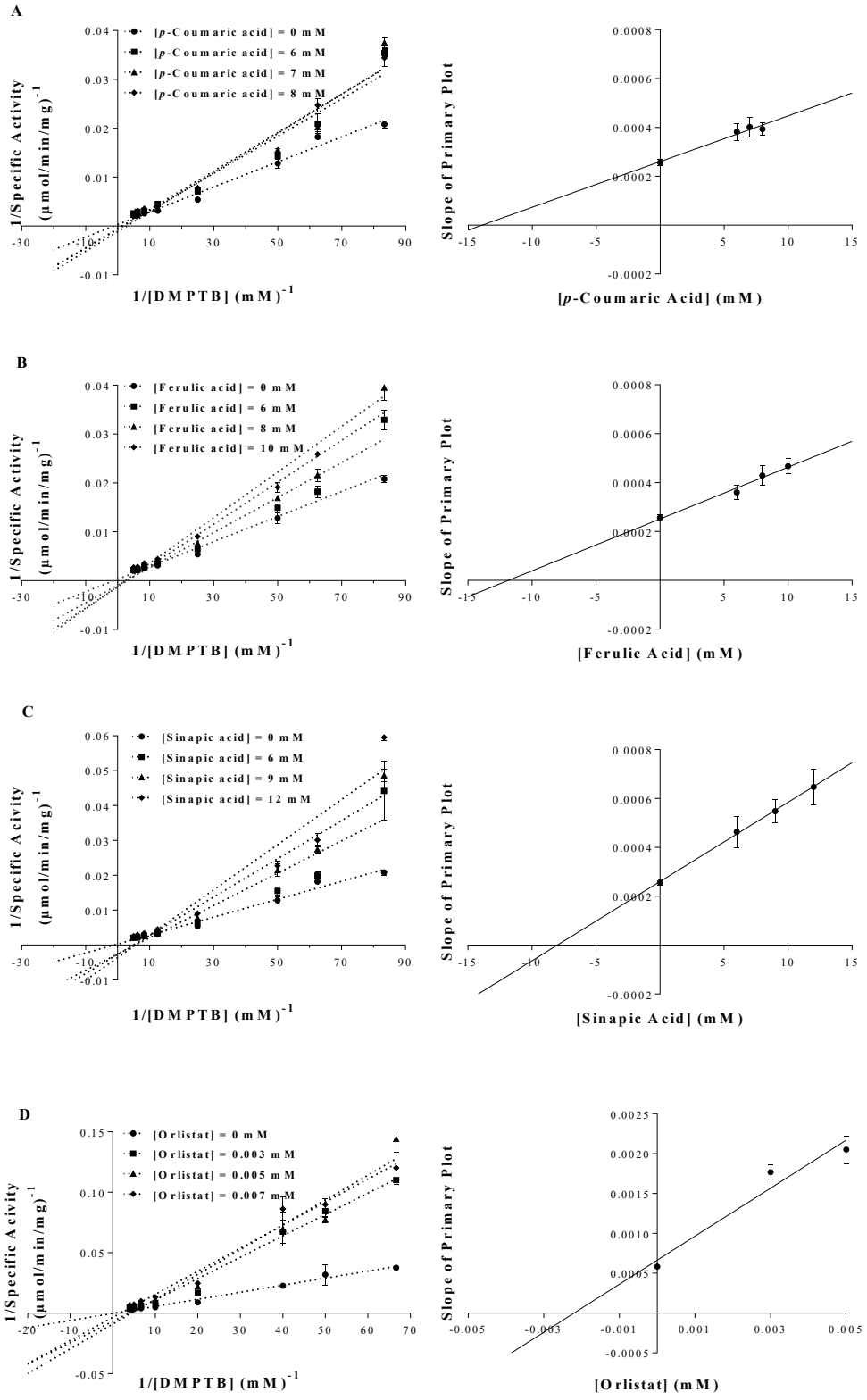

Figure 2 Lineweaver-Burk plots (left) and secondary Lineweaver-Burk plots (right) of lipase reaction with (**A**) *p*-coumaric acid, (**B**) ferulic acid, (**C**) sinapic acid and (**D**) orlistat. Each point was mean±standard deviation (SD) of triplicate assays.

The secondary Lineweaver-Burk plots were plotted by various substrate concentrations and the reciprocals of corresponding lipase specific activity with additional inhibitors (Figure 2, Table 3). The inhibitory constant (K_i) value was estimated from secondary Lineweaver-Burk plot, which was derived from the primary Lineweaver-Burk plot. The K_i is the dissociation constant of the enzyme-inhibitor intermediate or the enzyme-substrate-inhibitor intermediate, indicating effectiveness of interaction between enzyme and inhibitor. A small K_i value indicates a strong binding affinity of enzyme and inhibitor, whereas a large K_i value indicates a weak binding affinity. As results, the K_i of orlistat was 2.2 μM, while the K_i values of sinapic acid, ferulic acid and *p*-coumaric acid were 8.02, 11.82 and 13.90 mM, respectively. Based on these results, sinapic acid was the most effective inhibitors among all investigated phenolic acids, but was less effective than orlistat.

On the other hand, among flavonoid standards (myricetin, luteolin, isorhamnetin, quercetin, hesperitin, kaempferol and apigenin), it was found that both green and red bird chili contained no flavonoids. Previous research reported that other chili peppers in *C. frutescens* species seemed to exhibit a fair amount. Malaysian bird chili (*C. frutescens*) showed the highest content of flavonoids (1663.0 mg/kg dry weight), comparing to other edible plants[18]. The flavonoids in Malaysian bird chili (*C. frutescens*) comprised of myricetin (236.0 mg/kg), quercetin (392.0 mg/kg) and luteolin (1035 mg/kg)[18], which was undetected in bird chili in our experiment. Generally, natural flavonoids are mostly found in complex form with sugar (glycosides) or organic compounds. Hence, flavonoids in bird chili might be in low content or existed in bound form with other compounds.

Therefore, it is highly possible that the types and contents of phenolic acids play a great impact on anti-lipase property of chili peppers. External factors including fruit lengths, fruit widths, fruit pedicel lengths, fruit wall thicknesses, fruit weights and fruit color are hypothesized to have little effect on lipase inhibition. These observations were corresponded to the previous report on *C. annuum var. acumination*[23,24]. It was found that different sizes of chili pepper in *C. annuum var. acumination* did not affect total carotenoids, capsaicinoids content, flavonoids content and antioxidant activities. However, large chili peppers seemed to exhibit lower total phenol than its smaller counterpart. Nevertheless, this conclusion required more scientific evidences from other chili pepper sources to confirm this hypothesis.

Conclusion

Thai local chili peppers, both of *C. annuum* and *C. frutescens*, are cultivated with various types (banana pepper, spur pepper, yellow pepper, cayenne pepper, and bird chili), colors (green, yellow, orange and red), and shape (trigular, elongate and round). Moreover, chili peppers contain bioactive compounds such as flavonoids and phenolic acids that promote health benefits such as anti-obesity properties through key enzyme as lipase inhibitions. As result, all ethanolic (70% (v/v) ethanol) extracted Thai local chili peppers (23 samples) inhibited lipase activities with different degrees of inhibition. Green bird chili (*C. frutescens*) was found to exhibit the highest anti-lipase activity (49% inhibition), while red banana pepper exhibited the lowest (8% inhibition). The green bird chili with the highest anti-lipase activities was further investigated regarding their flavonoids and phenolic acids. Green bird chili and red bird chili contained no flavonoids but contained phenolic acids including *p*-coumaric acid, ferulic acid and sinapic acid. Green bird chili was found to exhibit the highest content of ferulic acid. Thus, high content of ferulic acid and its effectiveness might be a reason for the highest lipase inhibitory activity being detected in green bird chili. The knowledge on anti–obesity properties through key enzymes inhibitions of commonly consumed Thai chili peppers is useful for future development of functional foods, nutraceuticals or dietary supplements. Besides, the information on Thai local chili pepper will support consumption of local agricultural production for health maintaining purpose.

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