



Research Article

Kinetic Behaviour of Pancreatic Lipase Inhibition by Ultrasonicated *A. malaccensis* and *A. subintegra* Leaves of Different Particle Sizes

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Abstract

Gallic acid and quercetin equivalent were determined in the crude extract of matured leaves *Aquilaria malaccensis* and *Aquilaria subintegra*. The leaves of both *Aquilaria* species were dried at 60 °C for 24 hours, ground and sieved into particle size of 250, 300, 400, 500, and 1000 µm. Then, each particle size of leaves was soaked in distilled water with a ratio of 1:100 (w/v) for 24 hours and undergoes the pre-treatment method by using ultrasonicator (37 kHz), at the temperature of 60 °C for 30 minutes. The crude extracts were obtained after about 4 hours of hydrodistillation process. The highest concentration of gallic acid and quercetin equivalent was determined in the crude extract from the particle size of 250 µm. The kinetics of pancreatic lipase inhibition was further studied based using the Lineweaver-Burk plot, wherein the concentration of *p*-NPP as the substrate and pancreatic lipase were varied. Based on the formation of the lines in the plot, the crude leaves extract of both *Aquilaria* species exhibit the mixed-inhibition on pancreatic lipase, which indicates that in the reaction, the inhibitors were not only attached to the free pancreatic lipase, but also to the pancreatic lipase-(*p*-NPP) complex. The reaction mechanism was similar to non-competitive inhibition; however the value of dissociation constant, K_i , for both inhibition pathways was different. The inhibition shows an increment in Michaelis-Menten constant (K_m) and a reduction in the maximum pancreatic lipase activity (V_m) compared to the reaction without *Aquilaria spp.* crude extracts (control). This proved that the inhibition occurred in this reaction. Copyright © 2020 BCREC Group. All rights reserved

Keywords: *Aquilaria*; Gallic acid; Kinetic inhibition; Pancreatic Lipase; Quercetin

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1. Introduction

The issue of obesity and weight gain has become a priority now as it involves a person's level of health, from which critical illnesses can

stem from these problems. The prevalence of obesity and weight gain related diseases has encouraged various research institutions and biopharmaceutical companies to study and produce the weight-loss drugs. However, the demand for natural medicine is higher than chemical-based materials because awareness of the dangers and side effects that will be encountered. Hence, the needs for further development of weight-loss

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and obesity natural products are becoming more and more urgent. Nowadays, people are more concerned about the fat content in their body because for them, this is a major cause towards obesity and weight gain. Due to this matter, pancreatic lipase inhibitors have received more and more attention, since pancreatic lipase is a digestive enzyme produced by stomach and pancreas, which primarily function to break down fats into smaller molecules that can be easily absorbed and digested by intestines. Nevertheless, in theory, too much pancreatic lipase activity could be irritating because it could lead to obesity.

Alternatives found to slow down this enzyme activity is by taking the synthetic inhibitors such as anti-obesity drugs which have been deeply studied over decades. Last 13 years ago, FDA has approved four weight loss drugs which are Orlistat, Contrive, Belviq and Qsymia. There are only three drugs were approved by the FDA as adjunctive therapy for chronic weight management which are Orlistat that was approved in 1999, Belviq and Qsymia, both were approved in 2012 [1]. These clinical medications manipulate body weight by increasing energy expenditure, suppressing appetite, or inhibiting pancreatic lipase to decrease lipid absorption in the intestine [2]. Thus, as of September 2013, only Orlistat that has been approved by FDA and EMA (European Medicines Agency) for chronic weight management which able to inhibit gastrointestinal lipases in reducing fat absorption and it became the only FDA-approved weight loss drug that is available without a prescription [3]. The drugs are potential to decrease weight but possible side effects are always a big public health concern in new drug product development. However, Orlistat has a number of safety issues, including hepatotoxicity, nephrotoxicity, pancreatitis, kidney stones and the most common adverse effect is steatorrhea [3]. The risks also include severe liver damage, acute pancreatitis, acute renal failure, and pre-cancerous colon lesions. Other than that, Orlistat also interfere the fat-soluble vitamin absorption, which could cause a transient vitamin A, D, E, or K deficiency [4]. As conclusion, due to the modest efficacy, undesirable adverse effects, and serious health risks given by orlistat has highlighted the lacks of using Orlistat which later emphasizing the needs for other obesity drug options.

Therefore, traditional herbal medicines were believed in being able to suppress appetite and promote weight loss. These natural materials could be relatively more economical with no

toxic side effects when compared with the synthetic drugs. Natural products have potential in the treatment of obesity and it is still largely unexplored [5]. By exploring the potential of *Aquilaria sp.*, it might be an excellent alternative strategy for the development of safe and effective obesity control therapies. World Health Organization (WHO) declares that about 20000 plants are used for medical purposes and over 80% of world's population are using them for their primary health care [6]. This can be seen from the previous studies that explored anti-obesity compounds derived from green, white and black tea leaves which later become very popular functional food to be consumed [7]. There were findings proven that strong pancreatic lipase inhibitory activity were obtained for the in-vitro anti-lipase and antioxidant assays using crude ethanolic extracts from 30 plants grown in Oaxaca, México [8]. Strong inhibition of pancreatic lipase and pancreatic cholesterol esterase activities, as well as the inhibition of cholesterol micelle formation, and bile acid binding was also was proven by using mulberry leaf extract [9]. Thus, the results obtained by researchers proven that plant is able to help in controlling obesity.

Plant-derived lipase inhibitors have become potential research hotspots compared with chemically synthesized lipase inhibitors. Although it is difficult to determine the active components in the plant-based product sell in the market, but for consumer this product is relatively cheap, safe and reliable and known to be an effective slimming product, which makes the plant-based product is in demand. Therefore, this research explored the potential of two *Aquilaria sp.* namely *A. malaccensis* and *A. subintegra* as the pancreatic lipase inhibitor. Moreover, as up-to-date, it is nowhere to be found any studies on these species as the inhibitor for pancreatic lipase. The crude extract was obtained from hydrodistillation of ultrasonicated *A. subintegra* and *A. malaccensis* leaves for different particle size of 250, 300, 400, 500, and 1000 μm . Prior to extraction process, soaking and ultrasonication methods were introduced because it were claimed by most of researchers that both soaking and ultrasonication process were found to increase the specific surface area of the prepared samples while decreasing the average particle size and enhanced the yield of gallic acid and quercetin produced during extraction process [10–12]. The content of gallic acid and quercetin equivalent in the crude extracts were deter-

mined and the inhibitory studies were done.

Research on lipase inhibition by plant-based products has widely done and some of the research shows obvious inhibitory effects [13]. Yet, due to the low content of active ingredients, complicated in extraction procedures and low recovery rate, which make it cannot be produced in large quantities. Consequently, only few of them had undergo the clinical stage. This is a major drawback in commercialization of lipase inhibitors derived from edible plants. One of the suggestion is to study the action mechanism of natural compounds on pancreatic lipase, while the high-activity pancreatic lipase inhibitors are still continuously screened [13]. Thus, this research also emphasis on the reaction mechanism in inhibition of pancreatic lipase by *A. malaccensis* and *A. subintegra* leaves crude extract, wherein the activity of pancreatic lipase inhibitor was determined throughout the reaction. This research is one of the fundamental works for future studies on the natural obesity treatment, which is a new direction of the key research in the lipase inhibition mechanism field. It is also expected that the scientific data obtained would be beneficial towards the global development of agricultural and healthcare.

2. Materials and Methods

2.1 Drying, Milling, Sieving and Pretreatment of Leaves

The matured leaves of *A. malaccensis* and *A. subintegra* were collected from a farm in Selangor. The leaves were washed thoroughly and left to dry at room temperature before being dried in the oven (Memmert) at 60 °C for 24 hours. The dried leaves were milled by using Mastar (MAS-160BL (A)-1) blender to obtain fine powder. Next, the ground leaves were sieved into uniform particle sizes of powdered leaves at 250, 300, 400, 500 and 1000 µm. Then, each of particle size of *A. malaccensis* and *A. subintegra* powdered leaves were soaked for 24 hours in distilled water with a ratio of 1:100 (w/v) at room temperature. After soaking process, all particle sizes of leaves were ultrasonicated in a NEXXsonics NS-A-18H ultrasonicator. This pretreatment process was done at 30 minutes for each sample with a frequency of 37 kHz at 60 °C.

2.2 Hydrodistillation of Crude Extract

The pre-treated *A. malaccensis* and *A. subintegra* leaves was hydrodistilled by using a TOPS MS-6 heating mantle at constant atmos-

pheric pressure and at 70 °C until a sufficient amount of hydrodistillate was obtained (basically in a range of 300 to 400 mL). Then, the extracted leaves were evaporated under reduced pressure at 40 °C to get a concentrated product by using a Heidolph rotary evaporator. The crude leaves extract was kept in the refrigerator for further analysis and experiment in order to prevent any microbe from breeding through the samples.

2.3 Determination of Total Phenol Contents (TPC)

The total phenolic content was determined by using Folin-Ciocalteu method [14–17]. The mixture of 0.2 mL of leaves extract and 0.2 mL of Folin-Ciocalteu reagent were left for 4 minutes, in order to allow the reaction before it was added with 1 mL of 15% Na₂CO₃. After Na₂CO₃ was added, the mixture was allowed to stand for another 2 hours at room temperature. Then, the absorbance was measured at the wavelength of 760 nm. The absorbance was then used to obtain the concentration of gallic acid equivalent, by using the equation of gallic acid calibration curve that was constructed from the absorbance and concentration of gallic acid in the dilutions prepared from gallic acid stock solution. The stock solution was prepared by dissolving 100 mg of dry gallic acid in 2 mL of ethanol and diluted to volume with water in a 100-mL volumetric flask. Dilutions of this stock solution were done to have a range of gallic acid concentration between 0 and 1 mg/mL. The absorbance of each dilution solution was taken after Folin-Ciocalteu method was carried out and the plot of absorbance vs. gallic acid concentration was constructed. The results were expressed as mg/ml of gallic acid equivalent (GE). All readings were triplicated and the average of the readings was taken.

2.4 Determination of Total Flavonoid Contents (TFC)

The content of total flavonoids was determined by using the aluminium chloride (AlCl₃) assay [18]. The amount of 0.5 mL of leaves extract, 0.1 mL 10% AlCl₃, 0.1 mL of potassium acetate, and 4.3 mL of deionized water were mixed. Then, the mixture was incubated for 30 minutes at room temperature. The absorbance was then measured at the wavelength of 415 nm using spectrophotometer. The absorbance was then used to obtain the concentration of quercetin equivalent, by using the equation of quercetin calibration curve that was construct-

ed from the absorbance and concentration of quercetin in the dilutions prepared from the quercetin stock solution. The stock solution was prepared by dissolving 100 mg of dry quercetin in 2 mL of ethanol and diluted to volume with water in a 100 mL volumetric flask. Dilutions of this stock solution were done to have a range of quercetin concentration between 0 and 1 mg/mL. The absorbance of each dilution solution was determined by using the AlCl_3 method and the plot of absorbance vs. quercetin concentration was constructed. The results were expressed as mg/ml of quercetin equivalent (QE). All readings were triplicated and the average of the readings was taken.

2.5 Pancreatic Lipase Inhibition Reaction

Inhibition activity of pancreatic lipase was studied via spectrophotometric analysis [19,20]. Crude porcine pancreatic lipase (PPL) from Sigma (USA) was suspended in tris-HCl buffer (pH 7.4) with 2.5 mmol NaCl to give a concentration of 200 U/mL of pancreatic lipase. Then, *p*-nitrophenyl palmitate (*p*-NPP) was dissolved in water as pancreatic lipase substrate. Next, 1 mL of crude leaves extracts were mixed with 1 mL of enzyme suspension, 3 mL of substrate solution and 6 mL of tris-HCl buffer. The reaction was carried out in water bath at temperature of 37 °C for 30 minutes. After the reaction, 1 mL of acetone and ethanol mixture at a ratio of 1:1 was added in order to stop the PPL activity. The absorbance was measured by using spectrophotometer at wavelength of 410 nm. The absorbance was used to obtain the amount of *p*-nitrophenol (*p*-NP) liberated by using the equation from *p*-NP calibration curve. The calibration curve was constructed from the absorbance and concentration of *p*-NP at the range from 0 to 1000 $\mu\text{mol/mL}$. The readings were triplicated and averaged. The standard PPL activity which was without *Aquilaria* crude extract and the PPL activity of sample with *Aquilaria* crude extract were calculated using Equation (1).

$$\text{PPL Activity (PPLA)} = \frac{[C_{p\text{-NP}}]}{t_R} \quad (1)$$

Where $[C_{p\text{-NP}}]$ denotes of *p*-nitrophenyl released from *p*-NPP by 1 mL of PPL at 37 °C (μmol) and t_R denotes of the reaction time (min). Then, the percentage of inhibition (PI) was determined by using Equation (2).

$$\text{PI (\%)} = \frac{[\text{PPLA}_{w/o}] - [\text{PPLA}_w]}{[\text{PPLA}_{w/o}]} \quad (2)$$

Where $[\text{PPLA}_{w/o}]$ denotes of the standard PPL activity without *Aquilaria* crude extract ($\mu\text{mol/mL.min}$) and $[\text{PPLA}_w]$ denotes of the sample PPL activity with *Aquilaria* crude extract ($\mu\text{mol/mL.min}$).

2.6 Evaluation of Pancreatic Lipase Inhibition Kinetics

The kinetics analysis of PPL activity inhibited by *A. malaccensis* and *A. subintegra* leaves crude extracts was determined by using the graphical method via double reciprocal (Lineweaver-Burk) plots. The plots were constructed at different *p*-NPP concentration varying from 100 to 1000 $\mu\text{M/mL}$ for the PPL reaction with and without *Aquilaria* crude extract (control). The mode of inhibition was determined by looking at the pattern of interception and crossing of linear lines for the reciprocal data of PPL activity with and without inhibition vs. *p*-NPP concentration. All kinetic parameters which are the Michaelis-Menten constant (K_m) and the maximum reaction rate or enzyme activity (V_m), were obtained from the reciprocal of Michaelis-Menten Equation (3), which given by Equation (4), wherein the V_m was calculated from the interception at y-axis and K_m was calculated from the slope of the linear graph. The inhibition constant (K_i) was calculated by substituting K_m and V_m in the Michaelis-Menten kinetic equation (Equation 5), which was modified by taking the additional terms of K_i in the reaction into account when the mode of inhibition has been identified from the Lineweaver-Burk plot.

$$V = \frac{V_m [S]}{K_m + [S]} \quad (3)$$

$$\frac{1}{V} = \frac{K_m}{V_m} \frac{1}{[S]} + \frac{1}{V_m} \quad (4)$$

3. Results and Discussion

3.1 Content of Gallic acid and Quercetin in Ultrasonicated *Aquilaria* Leaves Crude Extract of Different Particle Sizes

The content of gallic acid and quercetin in *A. malaccensis* and *A. subintegra* crude extracts of different particle size of ultrasonicated leaves is shown in Figure 1. It was found that, the highest concentration of gallic acid and quercetin determined in both *Aquilaria* leaves crude extracts was at the particle size of 250 μm . This indicates that the total phenolic and flavonoid were significantly increased with decreasing particle size [21]. The particle size re-

duction of material from plant has turned into a fundamental viewpoint, where it has a significant effect in the extraction of active compounds, in which it provides a shorter mass transfer distance for the extraction solvent to travel and also more surface area were available for molecular transport [22–24]. It was also found that the extensive mass transfer of solute between phases also decreased the time for maximum phytochemical content to be extracted [25]. Furthermore, sample with larger particle size will have a smaller surface area which would restricted the solubility of water soluble components and led to decrease in the values of total phenolic and flavonoid content [26]. The aid of pretreatment method by using ultrasonication also contribute a significant effect, where the current particle size of leaves was reduced proportionally to the duration of time in this process, which leads to a reduction of particle diameter resulted by the cavitation energy generated by ultrasound, that raised local pressure changed and shifted in liquid which resulting in damaged on the particle [27]. There-

fore, optimization efforts on the ultrasonication process, specifically on the reduction of particle size, can be rationally developed with the presence of the quantification of such heuristic rules for each plant source [25]. The sample matrix, the particle size and the extraction technique also were strongly influence the phenolic and flavonoid extraction [28]. It was found that the concentration of gallic acid and quercetin equivalent in *A. subintegra* crude extract were 10% and 20% higher than the concentration of gallic acid and quercetin equivalent in *A. malaccensis* crude extract.

3.2 In-vitro Inhibitory effect of Ultrasonicated Aquilaria Leaves Crude Extract at Different Particle Sizes on Pancreatic Lipase

Figure 2 shows the percentage inhibition of porcine pancreatic lipase (PPL) by *A. malaccensis* and *A. subintegra* leaves crude extract that was calculated by using Equation (2) for particle sizes range of 250, 300, 400, 500, and 1000 μm . The highest percentage of PPL inhibition was given by the crude extract from leaves with particle size of 250 μm for both *Aquilaria* species. The percentage of PPL inhibition increased as the content of gallic acid and quercetin equivalent in the crude extract of different particle size increased. The result is in line with research done using green tea powders, wherein the inhibition depends on the content of phenolic and flavonoid of different particle size in the leaves [29]. The results obtained also revealed that antioxidant activity was dependent on particle size of powders [29]. Thus, it shows that smaller particle size leads to higher percentage of pancreatic lipase inhibi-

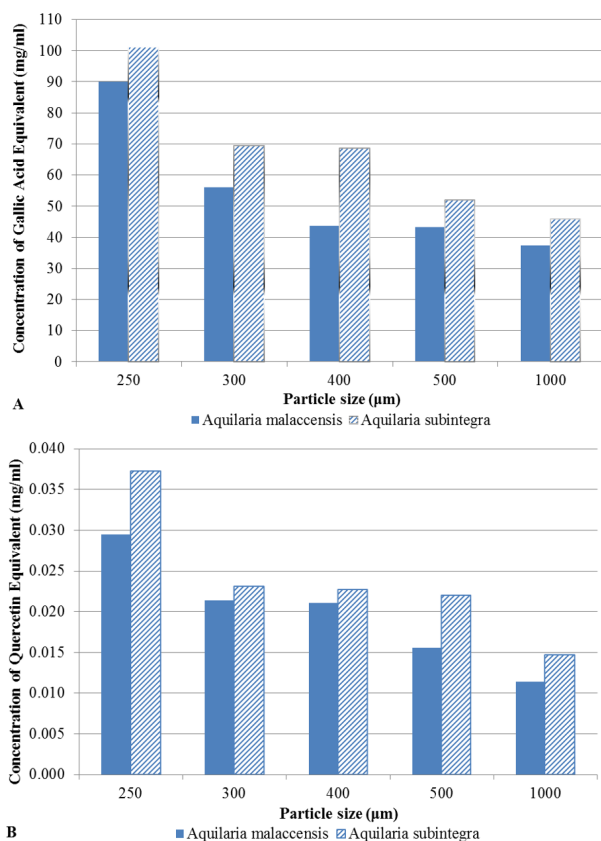


Figure 1. A) Gallic Acid and B) Quercetin concentration in the crude extract of *A. malaccensis* and *A. subintegra* at different particle sizes of leaves after ultrasonication process at 60 °C for 30 minutes.

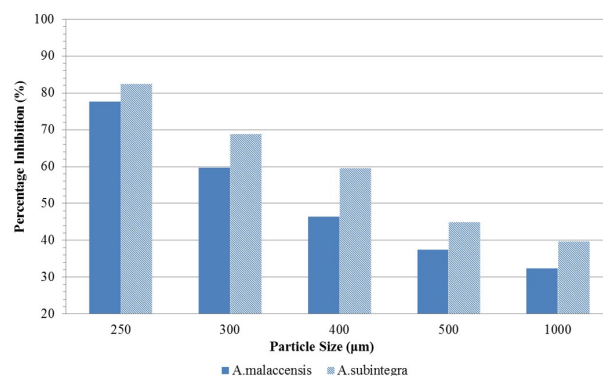


Figure 2. Percentage Inhibition of PPL by *A. malaccensis* and *A. subintegra* leaves crude extract for different particle sizes.

tion, due to the total phenolic and flavonoid content in the sample. The percentage of PPL inhibition was higher for *A. subintegra* compared to *A. malaccensis*, due to the content of gallic acid and quercetin equivalent were higher in the *A. subintegra* crude extract.

3.3 Kinetic Inhibition of Ultrasonicated Aquilaria Leaves Crude Extract at Different Particle Sizes

The mode of PPL inhibition exhibits by the Lineweaver-Burk plots of different particle size

of ultrasonicated *A. malaccensis* and *A. subintegra* leaves was found to be mixed-inhibition (Figures 3 and 4), which shows that *Aquilaria* extract was able to bind to free PPL and also to PPL-*p*-NPP complex. Based on the mode of inhibition identified, the overall inhibition reaction mechanism of mixed-inhibition featured by *A. malaccensis* and *A. subintegra* leaves crude extract is shown in Equation (5). Based on the reaction mechanism, both gallic acid and quercetin equivalent in *Aquilaria* crude extracts bound to PPL and to PPL-*p*-NPP complex, and there were possibilities for these in-

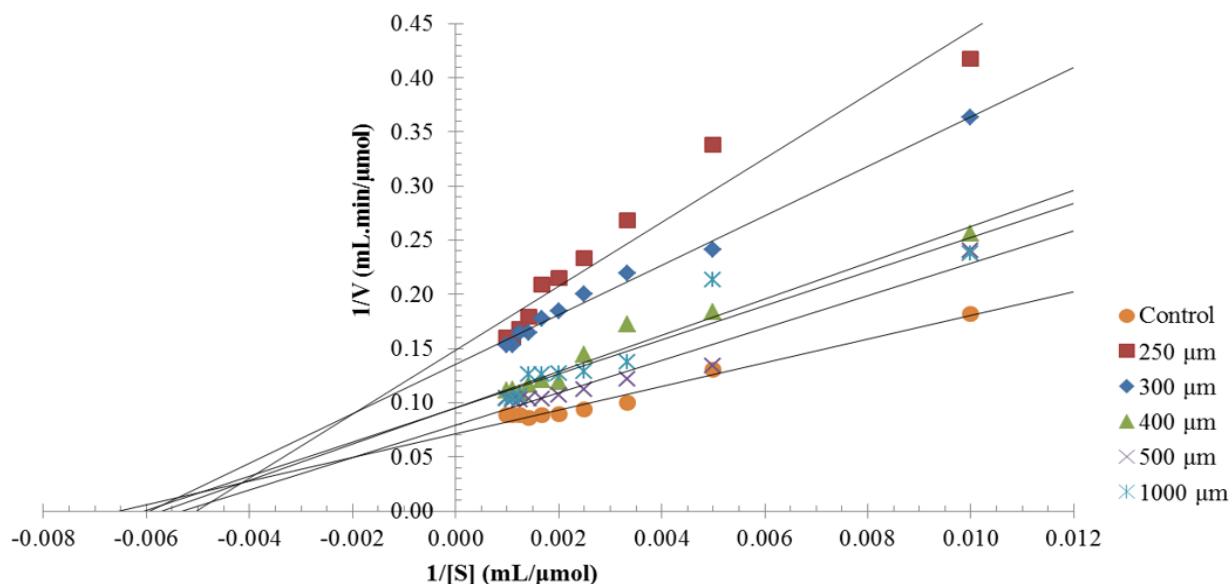


Figure 3. Lineweaver-Burk plots of PPL with and without *A. malaccensis* leaves crude extract for particle size of 250, 300, 400, 500 and 1000 μm .

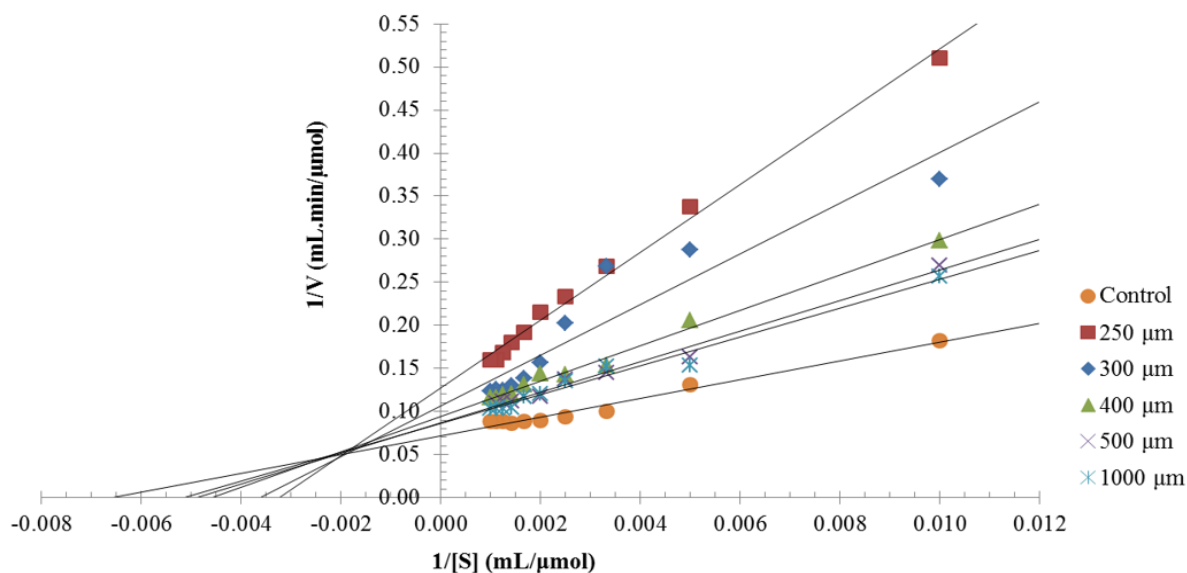


Figure 4. Lineweaver-Burk plots of PPL with and without *A. subintegra* leaves crude extract for particle size of 250, 300, 400, 500 and 1000 μm .

Table 1. Kinetic parameters of inhibition reaction by *A. malaccensis* and *A. subintegra* leaves crude extract at different particle sizes.

Species	Particle size (μm)	Galic Acid Equivalent Concentration (mg/ml)	Quercetin Equivalent Concentration (mg/ml)	Linear Line Equation	K_m (μM)	V_m ($\mu\text{mol/ml.min}$)	K_{ia} , Gallic Acid	K_{ih} , Gallic Acid	K_{ia} , Quercetin	K_{ib} , Quercetin
No Inhibitor (Control)	N/Z	0	0	$y = 10.904 x + 0.076$	81.87	12.67	N/A	N/A	N/A	N/A
<i>Aquilaria malaccensis</i>	250	89.991	0.029	$y = 29.548 x + 0.1483$	199.24	6.74	818.66	1204.48	0.15	0.22
	300	56.066	0.021	$y = 22.857 x + 0.1353$	168.94	7.39	735.98	879.73	0.16	0.19
	400	43.701	0.021	$y = 16.733 x + 0.0956$	175.03	10.46	1075.82	1651.91	0.29	0.45
	500	45.337	0.016	$y = 14.959 x + 0.0795$	188.16	12.58	1555.99	4960.50	0.30	0.96
	1000	37.433	0.011	$y = 15.728 x + 0.0949$	165.73	10.53	1094.70	1454.62	0.19	0.25
<i>Aquilaria subintegra</i>	250	101.273	0.037	$y = 39.383 x + 0.1272$	309.61	7.86	661.10	1788.40	0.14	0.37
	300	69.511	0.023	$y = 29.473 x + 0.1062$	277.52	9.42	634.41	1875.31	0.12	0.35
	400	68.750	0.023	$y = 20.526 x + 0.0944$	217.44	10.59	1086.31	2726.32	0.20	0.51
	500	51.988	0.022	$y = 17.777 x + 0.0868$	204.80	11.52	1103.87	3024.49	0.26	0.72
	1000	45.894	0.015	$y = 16.731 x + 0.086$	194.55	11.63	1130.16	2811.47	0.20	0.51

hibitors to bind at both state of PPL at the same time. In mixed-inhibition, the inhibitor is capable of binding to both the free enzyme and to the enzyme-substrate complex [30].

The linear line equation of Lineweaver-Burk plot (Equation 4) is the reciprocal of Michaelis-Menten Equation (3), wherein it can be written as Equation (6) to represent the overall PPL inhibition reaction by *Aquilaria* extract. The kinetic parameters which are the Michaelis-Menten constant (K_m), maximal velocity (V_m) and inhibition constant (K_{ia} and K_{ib}) for the mixed-type of inhibition reaction were calculated by using Equation (4) and (6). By referring to the overall reaction mechanism of *Aquilaria* extract shown in Equation (5), K_{ia} is the inhibition constant for binding of *Aquilaria* extract to the PPL and K_{ib} is the inhibition constant for binding of *Aquilaria* extract to the PPL-*p*-NPP complex. All calculated values of kinetic parameter were tabulated in Table 1.

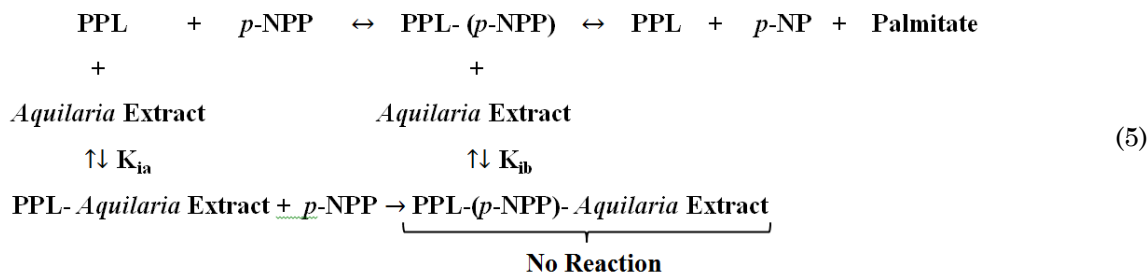
Based on the linear line equation obtained from the Lineweaver-Burk plots (Table 1), the interception at y-axis of the plot presented the value of $1/V_m$, where V_m was calculated from this reciprocal value. The slope of the graph presented the value of K_m/V_m , which is also known as specificity time [31]. Thus, the value of K_m was calculated by substituting the value of V_m which was formerly obtained from the y-axis interception. Therefore, in inhibition study, the K_m and V_m in Equation (4) was referred to the K_m and V_m value of inhibition, which can also be denoted as $K_{m,app}$ and $V_{m,app}$. In inhibition reaction, Equation (6) shows that the slope of K_m/V_m was decreased by a factor of $\{1+[Aquila\ Extract]/K_{ia}\}$, due to the inhibitory effect given by *Aquilaria* extract. In order to show that K_m and V_m were affected by *Aquilaria* crude extract and not by *p*-NPP, thus Equation (4) and Equation (6) was correlated and rearranged to obtain Equation (7) and (8), in which later it can be used to calculate K_{ia} and K_{ib} , where [*Aquilaria* Extract] was the concentration of gallic acid and quercetin equivalent in the *Aquilaria* crude extract.

The value of V_m also decreased because *Aquilaria* crude extract was proficient to prevent catalysis regardless of whether *p*-NPP was attached to PPL during the state of PPL-*p*-NPP complex (Equation 5). With mixed-type inhibitors in the reaction, the K_m varied with the relative values of K_{ia} and K_{ib} , wherein the value of K_m increased as the K_{ia} or K_{ib} value decreased. Moreover, in mixed-inhibition, the dissociation constant of inhibitor binding at free PPL was

differed from the dissociation constant for binding at PPL-*p*-NPP complex. The value of K_i for an inhibitor is analogous to K_m for a substrate, where it reflects the strength of the interaction between the PPL and *Aquilaria* crude extracts at the state with or without *p*-NPP. A small K_i value reflects the tight binding of an inhibitor to an enzyme, whereas a larger K_i value reflects the weaker binding. The results found that the K_{ib} value was higher than K_{ia} , in which this indicates that the affinity of inhibitor bound to free PPL was higher than the binding of inhibitor to PPL-*p*-NPP complex, which makes the inhibitory effect stronger. Yet, according to the Federation of European Biochemical Societies, the case in which K_{ia} is lower than K_{ib} are known as predominantly competitive inhibition [32]. Furthermore, the presence of *p*-NPP on PPL has no influence on the ability of a mixed type inhibitor to bind to PPL because even though it does not have structural similarity to *p*-NPP, it was able to bind to both the free PPL and the PPL-*p*-NPP complex. Although the binding is away from the active site, it can still alter the conformation of the enzyme and reduce its catalytic activity due to changes in the nature of the catalytic groups at the active site [33].

Similar inhibition mode is also observed in the pancreatic lipase inhibition study done by Ong *et al.* using the extracts of *Eleusine indica* (*L.*) *Gaertner* [34]. It indicates the possibility of pancreatic lipase-substrate complex formation with the inhibitor binding at a distinct site from the active site resulting in reduction in complex affinity. Thus, this explained the increase in K_m for the inhibition of PPL by using the crude extract from ultrasonicated *Aquilaria* leaves. In the inhibition by *A. subintegra* crude extract, it was found that the effect of mixed-inhibition was a reduction in V_m and decrement in K_m as the particle size of leaves increased.

However, K_m for PPL inhibition by *A. malaccensis* crude extract increased for the particle size of 400 and 500 μm which indicates that the affinity between PPL and *p*-NPP was lower, regardless to the content of the gallic acid and quercetin equivalent in *A. malaccensis* crude extracts. The sudden increment of V_m at 500 μm of *A. malaccensis* leaves was due to the higher PPL activity at lower *p*-NPP concentration compared to PPL activity at higher *p*-NPP concentration (Figure 3). Thus, it can be concluded that a larger value of K_m shows a weak binding of substrate to enzyme [35]. The



$$\frac{1}{V} = \left(1 + \frac{[\text{Aquilaria Extract}]}{K_{ia}} \right) \frac{K_m}{V_m} \frac{1}{[p\text{-NPP}]} + \frac{\left(1 + \frac{[\text{Aquilaria Extract}]}{K_{ib}} \right)}{V_m} \tag{6}$$

$$V_{m,app} = \frac{V_m}{1 + \frac{[\text{Aquilaria Extract}]}{K_{ib}}} \tag{7}$$

$$K_{m,app} = K_m \frac{1 + \frac{[\text{Aquilaria Extract}]}{K_{ia}}}{1 + \frac{[\text{Aquilaria Extract}]}{K_{ib}}} \tag{8}$$

change in K_m also might varies, and it is depend on the relative values of inhibition constant at free enzyme (K_{ia}) and inhibition constant at enzyme-substrate complex (K_{ib}).

4. Conclusions

Based on the results obtained, it turns out that the smallest particle size of leaves which was 250 μm , that have been soaked for 24 hours with a ratio of 1:100 (w/v) and ultrasonicated at 60 $^{\circ}\text{C}$ for 30 minutes resulted the highest content of gallic acid and quercetin equivalent in *Aquilaria* crude extract, as well as giving the highest percentage of inhibition towards PPL. It was proven that smallest particle size provides a great effect in obtaining higher gallic acid and quercetin content in the crude extract. The lowest PPL activity was found in the inhibition reaction using *Aquilaria* crude extract from 250 μm particle size of leaves. The existence of gallic acid and quercetin equivalent in the crude extracts had contributed to the inhibition of PPL, with the highest percentage PPL inhibition of 82% for 1 mL of *Aquilaria* crude extract used. The percentage of PPL inhibition could be increased with increasing volume of *Aquilaria* crude extract used. Studies on kinetics were initiates by identifying the mode of inhibition presented by *Aquilaria* against PPL using Lineweaver-Burk kinetic plot. The mode of inhibition identified

for the sample with highest content of gallic acid and quercetin equivalent was mixed-inhibition. In this type of inhibition, the value of K_m was higher and V_m was lower compared to the value of K_m and V_m for the non-inhibited PPL. It indicates that this reversible inhibitor decreased the rate of PPL activity and also reduced the affinity between $p\text{-NPP}$ and PPL to react. Furthermore, it is believed that the pancreatic lipase has the other site than the active site for both the $p\text{-NPP}$ and inhibitor in *Aquilaria* crude extract to bind. The inhibition constant for mixed-inhibition were known as K_{ia} and K_{ib} , wherein these two constants had different values with one was higher to the other one. Lower value of K_{ia} indicates that the affinity between inhibitors in *Aquilaria* leaves crude extract and free PPL was higher. The inhibitor activity and kinetic parameters determined from *Aquilaria* spp. is expected to benefit in controlling obesity and also problems associated with excess weight. Besides, it can further increase the potential of widely planted and wildy grown *Aquilaria* species. It is expected that this study will extend the knowledge on enzyme inhibition kinetics and all related parameters that may able to help in identifying the level of effectiveness of an inhibitor that would also benefits the relevant research in this area.

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