Effect of Substrate Concentration and Reaction Time of *Aquilaria subintegra* Leaves Extract on Inhibition of Pancreatic Lipase

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Abstract. The purpose of this research are to recovery of pancreatic lipase inhibitor and to study the effect of using different concentration of substrate and reaction time on pancreatic lipase inhibitor. In this research, *Aquilaria subintegra* mature and fresh leaves was used as a sample. The research was conducted by using hydro-distillation with different concentrations, which are 100 µM, 200 µM and 300 µM and reaction times from 20, 40 and 60 minutes were studied. Based on the results obtained for the samples of phenol, flavonoid, gallic acid and quercetin were 49.30 µg/ml, 314.33 µg/ml, 12.94 µg/ml, and 5.15 µg/ml, respectively.

1. Introduction

Aquilaria also commonly known as agarwood, gaharu, aloeswood, eaglewood or Oudh is belonging to Thymelaeceae family. Aquilaria species mainly produces in Southest Asia, with Indonesia, Malaysia, Vietnam, Cambodia, Thailand, Laos and Papua New Guinea [1]. Aquilaria is fragrant resinous wood from tropical green forest and estimated of 20 species that located mainly in Asia [2]. Agarwood has been used as a traditional medicine used to treat aches, muscular pains, inflammation and anaphylaxis for over thousands of years. It's also very widely used for centuries as incense for religion purpose. Recently, agarwood has been widely used for research purposes in medical area that can treat many type of diseases. It reached with chemical constituents such as phenolic compounds, flavonoids, terpenoids, alkaloids, tannins and many more [3-5]. Agarwood can been formed through inducement process such as injury, cutting, pest or insect, disturbance, microorganism, fire, chemical or colonization. Recently, obesity problem has growing rapidly for bigger health crisis in the world every year. Obesity is when someone is overweight that can risk their health. Obesity occurs from over-eating habit especially an unhealthy diet and lack of exercise. The study found that more than 60% of men and 50% of women in the world were either overweight or obese. Obesity can lead to many health problems including heart disease, stroke, diabetes, high blood pressure, unhealthy cholesterol, cancers and many more.

There has an effective way that can prevent and solve the obesity problem which is inhibit fat absorption in human digestive system. The key for inhibit fat absorption is pancreatic lipase that inhibit the pancreatic lipase. Pancreatic lipase is an enzyme secreted from pancreas that hydrolyses and break down dietary fat molecules with converting triglyceride substrates found in ingested oils to mono-glycerides and free fatty acid. The free fatty acid can cause obesity problem with the formation of free fatty acid will contributing to insulin resistance that is major lead to obesity. There are many researchers that study about this anti-obesity by recovery of pancreatic lipase inhibitor from many

kinds of natural resources. Many plants have been reported to inhibit lipase activity which the presence of secondary metabolites such as polyphenols, benzopyrines whose members include flavonoids, saponins, and caumarins [6]. Thus, in this research is to recover the pancreatic lipase inhibitor. *Aquilaria subintegra* (*A.subintegra*) has rich in phytochemicals content as potential of pancreatic lipase inhibitor. The bark, stem and leaves of *A.subintegra* have compound of pancreatic lipase inhibitor. Hydro distillation technique can help to extract the *Aquilaria sp* in order to get the compound of pancreatic lipase inhibitor. The different of substrate concentration, reaction time and temperature will affect the recovery of pancreatic lipase inhibitor from extraction of *Aquilaria sp*. Hence, this project is recovery of the pancreatic lipase inhibitory from extraction of *A.subintegra* leaves and to determine the effect of different substrate concentration and reaction time use on pancreatic lipase inhibitory recovery. There are some compounds that can act as pancreatic lipase inhibitor that can solved people that have obesity problem. Hence, this research project for recovery of pancreatic lipase inhibitor compound from the extraction of *A.subintegra* leaves.

In this study, the compounds and its concentration that can act as pancreatic lipase inhibitor is determined. However, there are some parameters that can affect the recovery of pancreatic lipase inhibitors of *A.subintegra* leaves extract that are substrate concentration and reaction time for the extraction of *A.subintegra* leaves. Thus, for this research purpose is to determine what are the effects of substrate concentration and reaction time from *A.subintegra* leave extraction on inhibition of pancreatic lipase recovery.

2. Experimental

2.1. Materials

The material used for this research were mature and fresh *A.subintegra* leaves.

2.2. Preparation of Leaves Samples

The drying process of the leaves was done by using Memmert oven at 60°C. This step was important in order to remove any moisture present in the leaves. Next, the fresh sample of *A.subintegra* leaves is preferred in form of grinded form in order to provide larger surface area that increasing the rate of reaction between the samples molecules and the solvent molecules in the extraction process. The grinding process was done by using the Mastar (MAS-160BL(A)-I) blender for around 1 minute until the required size of leaves were obtained. The grinded leaves were then sifted through a 250 μm sieve in order to obtain the uniform size for further procedures.

2.3 Soaking

1. The dried fiber and also the powdered *A.subintegra* samples were soak in the distilled water to extract the grinded leaves samples [7]. It was done for 24 hours at room temperature. The ratio of the grinded leaves to distilled water was 1:100 g/mL.

2.4 Ultrasonication

All dried and soaked fiber and powdered *A.subintegra* samples will further enhance using ultrasonication. The samples were placed in a NEXXsonics NS-A-18H ultrasonicator (37 KHz) at 30 ^{o}C, and ultrasonication time was 30 minutes for each sample.

2.5. Hydro-Distillation

The dried and grinded leaves samples continue in hydro-distillation process. The heating process were done by using the heating mantle of TOPS MS-06 at the atmospheric pressure. The heating process was continuously until all essential oil was obtained at the receiving flask usually the sufficient amount of hydrodistillate were between 350 mL to 400 mL. This is to ensure all phenolic compounds has been distilled [3]. After that, the leaves extract was evaporated in order to ensure there is no more concentrated crude leaf extract which is more suitable for further testing by using a Heidolph rotary evaporator (Laborota 4000 efficient). After the extraction process end, the apparatus was let to cooled to room temperature before the sample was continued for extraction [8]. The extracted samples were transferred into the tight sample bottle kept in the room temperature.

2.6. Lipase Assay

The lipase from porcine pancreas (Merck KGaA) was prepared and suspended in tris-HCl buffer (pH 7.4) with sodium hydroxide, NaOH (2.5 mmol) to result in a 200 unit/mL lipase solution. Assay buffer was p-Nitrophenyl Phosphate (p-NPP) was dissolved in isopropanol to a concentration of 100 µmol and used as substrate and different substrate concentration was used that are 200 and 300 µmol. Next, 3 mL of each extracted leaves sample was mix with 1 mL of the enzyme suspension and 1 mL of substrate solution. 6 mL of tris-HCl buffer was added into the mixture. The mixture was mixed with hydrolytic reaction followed by incubating at 37°C for three different times, which are 20 minutes, 40 minutes and 60 minutes. The acetone-ethanol mixture with 1:1 ratio was added to stop the reaction before measure the absorbance value using Hach DR 2800 spectrophotometer at wavelength 405 nm. The leaf extract absorbance value was compared with control sample or blank sample. Hence, the percentage of inhibition was determined based on absorbance value obtain with compared with control sample.

2.7. Analytical Method

The samples are analyzed by using high performance liquid chromatography (HPLC) on a Waters Millennium 32®system. To detect the present of gallic acid and quercetin in the samples. The phenolic and flavonoid is analyzed by difference method that are using Total Phenol Content and Total Flavonoid Content method. The samples are using spectrophotometer to determine the value of absorbance by using 415 nm and 760 nm of wavelength [9], and the absorbance values were determined by averaging 3 readings for each sample.

2.7.1 Limits of detection and the standard curve

The limits of detection (LOD) were calculated from the relationship between the standard deviation (SD) and the slope, using the appropriate multiplier. The calibration curve of the flavonoid standard (5.0 to 100.0 µg/mL), phenol (5.0 to 80.0 µg/mL), gallic acid (0.5 to 3.0 µg/mL), and Standard quercetin (2.5 to 25.0 µg/mL) were determined from four to five concentration points over the range of concentrations, following the Lambert-Beer law. The slope and other statistics of the calibration curves were calculated by linear regression by using Microsoft Excel.

3. Results and Discussions

3.1. Volume of Sample after Pre-treatment

The best efficiency for volume for leaves extract after hydro-distillation should be between 350 ml to 400 ml. From Table 1, the volume of the sample (After Hydro-distillation) for mature and fresh leaves are not standardize is due to the variation of extraction parameters. The volume of leaves extract after evaporation should be in between 16 to 20 ml for mature and fresh leaves.

Table 1. Volume extract after pre-treatment for mature leaves and fresh leaves

Mature Leaves			Fresh Leaves		
Samples	**After Hydro-distillation (mL)**	**After Evaporation (mL)**	**Samples**	**After Hydro-distillation (mL)**	**After Evaporation (mL)**
A1	330	20	**B1**	342	20
A2	379	17	**B2**	347	18
A3	332	18	**B3**	343	20
A4	361	20	**B4**	345	18
A5	366	20	**B5**	334	16
A6	349	20	**B6**	345	20
A7	344	19	**B7**	361	20
A8	353	20	**B8**	362	16
A9	363	20	**B9**	340	16

3.2. Total Flavonoid Content

From Table 2, the highest concentration of flavonoid content calculated by using the standard curve of flavonoid is A8 which is 354.33µg/ml while the lowest is A3 which is 191µg/ml, this finding also support by Bahrani et al. 2014 [10] and Naef 2010 [11].

Table 2. Average absorbance value of total flavonoid content for mature leaves and fresh leaves

Mature leaves		Fresh Leaves	
Samples	**Absorbance**	**Samples**	**Absorbance**
A1	0.274	**B1**	0.242
A2	0.204	**B2**	0.272
A3	0.173	**B3**	0.212
A4	0.285	**B4**	0.257
A5	0.211	**B5**	0.259
A6	0.273	**B6**	0.236
A7	0.189	**B7**	0.224
A8	0.320	**B8**	0.195
A9	0.289	**B9**	0.284

While, the highest concentration of flavonoid content calculated by using the standard curve of flavonoid is B9 which is 314.33 µg/ml while the lowest is B8 which is 215.44 µg/ml Flavonoid content dry has higher than fresh leaves.

3.3. Total Phenol Content

From Table 3, the highest concentration of phenol content calculated by using the standard curve of phenol is A3 which is 58.22µg/ml while the lowest is A6 which is 21.70µg/ml. The highest concentration of phenol content calculated by using the standard curve of phenol is B9 which is 49.30 µg/ml while the lowest is B3 which is 21.26µg/ml, this finding also support by Bahrani et al. 2014 [10] and Naef 2010 [11]. Phenol content of dry leaves has higher compare to fresh leaves.

Table 3. Average absorbance value of total phenolic content for mature leaves and fresh leaves

Mature Leaves		Fresh Leaves	
Samples	**Absorbance**	**Samples**	**Absorbance**
A1	0.232	**B1**	0.236
A2	0.258	**B2**	0.242
A3	0.333	**B3**	0.163
A4	0.169	**B4**	0.250
A5	0.189	**B5**	0.202
A6	0.165	**B6**	0.277
A7	0.206	**B7**	0.213
A8	0.253	**B8**	0.213
A9	0.180	**B9**	0.292

3.4. The Effect of Substrate Concentration and Reaction Time

From Tables 4 and 5, the best inhibition is in lower substrate concentration and short reaction time for both dry and fresh samples. This may due to long reaction will lower the efficiency of the inhibitors.

Table 4. Average absorbance value of mature leave samples for 100, 200 and 300 µM substrate concentrations and percentage inhibition

Time (min)	**Concentration (µM Absorbance)**					
	100	**(%)**	**200**	**%**	**300**	**%**
20	0.215	31.5	0.221	29.6	0.238	24.2
40	0.276	12.1	0.247	21.3	0.246	21.7
60	0.265	15.6	0.269	14.3	0.255	18.8

Table 5. Average absorbance value of fresh leave samples for 100, 200 and 300 µM substrate concentrations and percentage inhibition

Time (min)	Concentration (µM Absorbance)					
	100	**(%)**	**200**	**%**	**300**	**%**
20	0.215	31.5	0.237	24.5	0.261	16.9
40	0.218	30.6	0.231	26.4	0.217	30.9
60	0.282	10.2	0.261	16.9	0.246	21.7

3.5. HPLC (High Performance Liquid Chromatography)

HPLC is one of the important widely used purification techniques for the isolation of natural products such as flavonoids, gallic acid, phenolic and quercetin compounds [12].

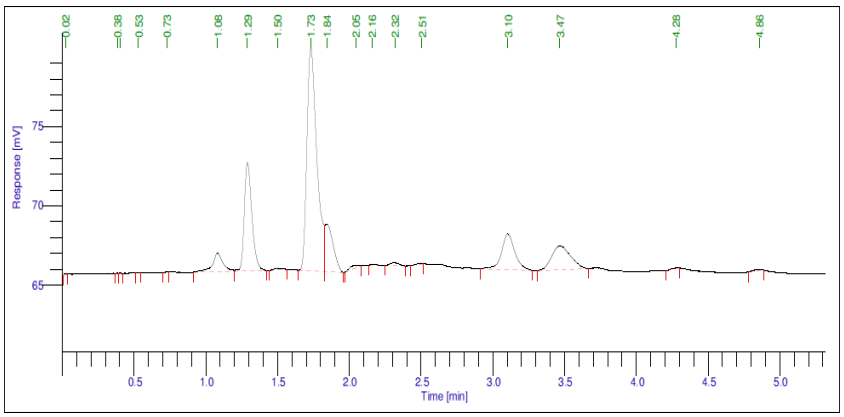

Figure 1. HPLC result for sample A5 sample

From the HPLC result of mature and fresh leaves samples from Figures 1 and 2, it shows the both samples mature leave (A5) and fresh leave (B5) contains garlic acid and quercetin compounds. Based on standard curve equation for garlic acid and quercetin compound the highest concentrations were obtained are 12.94 µg/ml and 5.15 µg/ml, respectively.

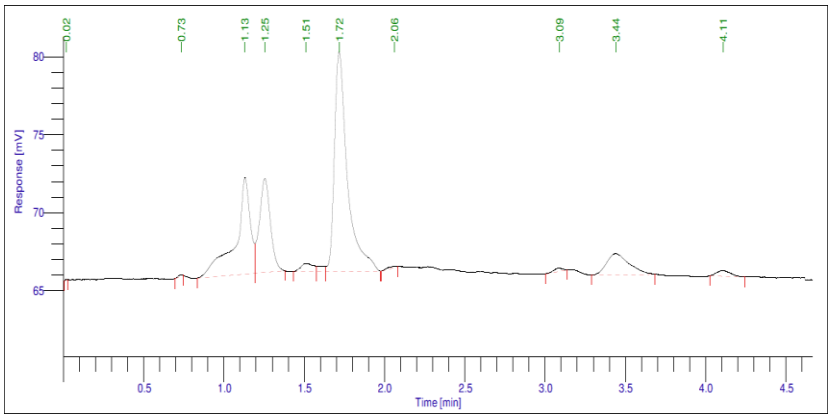

Figure 2. HPLC result for sample B5 sample

4. Conclusion

In this research, we have recovered the pancreatic lipase inhibitors from *A.subintegra* mature and fresh leaves extract. There contains phenol (49.30 µg/ml), flavonoid (314.33 µg/ml), gallic acid (12.94 µg/ml) and quercetin (5.15 µg/ml) from this research. Future work on this research will focus more about the potential of this leaves extract with more feasibility of different materials and technique to improving the efficiency of resource utilization.

Acknowledgements
The authors would like to acknowledge Faculty of Chemical Engineering, UiTM for providing analytical instruments, materials and support by researchers, academician, practitioners and staffs contributed towards understanding and thoughts and guidance.

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