Recovery of Lipase inhibitory compound from *Aquilaria Malaccensis* and *Aquilaria Subintegra* matured leaves extract via pretreatment using bath sonicator: Effect of ultrasonic time reaction

Nurul Farhana binti Mashuri, Prof. Dr. Ku Halim Ku Hamid, Miradatul Najwa Mohd Rodhi

Faculty of Chemical Engineering, Universiti Teknologi MARA

***Abstract*— Obesity has been arising from a day to day and has become a very complicated health issue worldwide that must be seriously taken. Obesity can be prevented by inhibiting pancreatic lipase that will block adsorption of fat. Aquilaria spp. (gaharu) is one of Malaysian treasures, rich in phytochemicals content in its resin. It was attentive to discover the miracle of this species which having a lot of health benefits including as a natural anti-obesity potent. This research was conducted to identify the effect of particle size on the inhibition activity at different ultrasonic time reaction. It was also carried out to determine the inhibitory effects of phytochemical compounds on pancreatic lipase that lead to the obesity prevention. The presence of phytochemical contents of both *A.malaccensis* and *A.subintegra* were observed. While the inhibitory effects on pancreatic lipase was examined using spectrophotometer analysis. The presence of phenolic and flavonoid compound in *A. Malaccensis* and *A.Subintegra* leaves extract. The parameters involved in the process were compound particle size of (0.25M, 0.5M, 1.0mM) and reaction time (30, 60, 90, 120 and 150 minutes) during inhibition of pancreatic lipase process. From the result obtained from using Mastersizer 2000E, the effect of ultrasonic reaction time on particle sizes are obtained which indicates the reduction of particle sizes across time. Next, inhibitory activity is determined by studying the effect of ultrasonic reaction on pancreatic inhibition activity. By getting those result, a new findings are obtains, which is the effect of particle sizes on inhibitory activities. The percentage of inhibition was higher at 90 minutes at the optimum temperature of 60 ^{o}C.**

Keywords: Phenolic compound, Aquilaria Malaccensis, Aquilaria Subintegra, Gallic acid, Quacertin

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I. INTRODUCTION

Aquilaria is a genus of fifteen species of plant in the *Thymelaeaceae*, inhabitant to southeast Asia. They can be found especially in the rain forests of Indonesia, Thailand, Cambodia, Laos, Vietnam, Malaysia, Northern India, the Philippines, Borneo and New Guinea. The trees grow to 6-20 m tall. The leaves are alternate, 5-11 cm long and 2-4 cm broad, with a short edged top and a full margin. The flowers are yellowish-green, the fruit is a woody capsule 2.5-3 cm long. [1] This resin is produced as a result of pathological or wounding processes. Some researchers prove that production of resin is a response to fungal infection. However, not all Aquilaria trees produce resin and it is kind of hard (or might be impossible) to judge from the outside of a tree whether or not it is infected. The only way to know is by cutting the trees. [2]

This species is one of the valuable products due to its very high demand in industries such as perfumery and pharmaceuticals. Use of agarwood has been known in many previous millennium years. People of different countries normally had different belief and credence on the use of this tree species. For example, the Egyptians are believed to have used agarwood incense as part of their death rituals more than 3,000 years ago. In Japan, agarwood is said to have arrived with Buddhism. In Vietnam age-old people also assigned to the use of agarwood in relation to travelling Buddhist monks.[2]

Obesity is one of diseases that can be prevented by inhibiting the production pancreatic lipase as to block fat absorption in the small intestine after being hydrolysed by pancreatic lipase pancreatic lipase. Pancreatic lipase is secreted to hydrolyze fat in the body into fatty acids which causing to the digestion and storage of fat. In the case of obesity, this is a bad impact to those people suffering from this disease. [3] From previous research, it is proven that gaharu species contain the lipase inhibitory compound, which is phytochemical compound such as polyphenol and flavonoid. In this research, the natural source of treatment for obesity is to be sourced from Aquilaria

subintegra and Aquilaria malaccensis matured leaves as they are known in the medicinal sector to provide a lot of health benefits to treat obesity, diabetes and cancer.

Common names of this species are agarwood, aloeswood, and eaglewood. They are also be called gaharu or karas for people in Malaysia and Indonesia as their vernacular names. [4] The focus of this research is to determine the effect of reaction time on recovery of pancreatic lipase inhibitor from *A.Malaccensis* and *A.Subintegra* matured leaves extract. Hydro distillation technique is used for extraction of phytochemical compound of inoculated *Aquilaria Malaccencis* and *Aquilaria Subintegra* as this technique is safe to operate, eco-friendly and cost effective [5].

II. MATERIALS AND METHODS

A. PREPARATION OF SAMPLE

i. Collection of Plant Materials

The leaves of A.Malaccensis and A.Subintegra that are used in this research are collected. The leaves that were collected were only matured leaves. The leaves then washed to be cleaned, as if there is presence of any dirt might affect the result of the experimental work. Also, the leaves were dried at room temperature before being dried using oven.

ii. Drying and Grinding

The leaves must be dried first, in order to remove moisture. The leaves were dried in Oven. The optimum temperature for this drying process was 60°C to 70 °C for 24 hours. After that, the dried leaves were ground with a grinder. The ground leaves were then sifted through a 250 µm sieve to obtain a uniform size for further procedures. To obtain more product, the leaves extracts were put in Mastar (MAS-160BL(A)-I) blender in 1 minute to result fine powder. The powdered leaves are used in this research. Both fiber residue and powdered leaves were used in this research.

iii. Pre-treatment of Leaves

a. Soaking

The powdered leaves is then been soaking with distilled water to extract the compound from the ground leaves. The soaking time was 24h at room temperature. The ratio of leaves to distilled water was 1:100g/mL. The same method is applied to both the powdered leaves of A.Malaccensis and A.Subintegra.

b. Ultrasonic Pre-treatment

Ultrasonication was performed on all dried and soaked fiber and powdered Aquilaria subintegra samples to further enhance leaf extraction. Ultrasonic pre-treatment of the leaves was done at frequency 37 kHz. The time of ultrasonication was increasing 30 minutes for each sample to determine the effect of ultrasonication on leaf extraction. The leaves are placed in NEXXsonics NS-A-18H ultrasonicator. The aim of this is to determine the effect of ultrasonication on leaf extraction.

iv. Hydrodistillation of Leaf Extract

Hydrodistillation is a process of boiling the samples of leaf extract by using distilled water. The process was done using round bottom flask with the temperature maintained at 70°C. Thus, the dried, ground and pretreated Aquilaria subintegra leaf extract is hydrodistilled using the TOPS MS-06 heating mantle at atmospheric pressure until a sufficient amount of the hydrodistillate is obtained, normally around 250 mL to 300 mL. This is to ensure all phenolic compounds in the mixture have been distilled in order to obtain accurate results in further procedures of the experiment. The distillate collected is expected about 90% of the volume extract. The sample then was evaporated using Heidolph rotary evaporator (Laborota 4000 efficient) to ensure a more concentrated crude leaf extract which is more suitable for testing. The samples were kept refrigerated before going to analyse tp prevent any microbial from breeding through the samples.

B. ANALYSIS OF EXTRACTED COMPOUNDS

i. MASTERSIZER 2000E

Identification of the active functional groups presence in inoculated Aquilaria essential oil alsocan be performed using a Mastersizer 2000E. The measuring range of this instrument is 0.1 to 100 microns. The detection system has the red color light Helium neon laser with forward scattering, side scattering, and back scattering. The optical alignment having an automatic rapid alignment system with dark reticle and multi-element alignment detector and beam wavelength is 633 nanometer. For a preparation of a 1g samples, the samples were dispersed in 50 mL isopropanol, IPA. Pasteur pipette was used for sampling. IPA is as the carrier fluid. Each measurement is repeated for 6 times and the samples inside the wet unit is not changing. [6]

ii. PANCREATIC LIPASE INHIBITION

Firstly, in the preparation of enzyme, the crude porcine pancreatic lipase (Sigma, USA) was suspended in 2.5 mmol of NaCl with tris-HCL buffer at pH 7.4 so that the concentration of the pancreatic lipase assay will become 200 units/ mL. Next, the p-NPL was utilized as pancreatic lipase substrate dissolved in water at 100 µM concentration. 1mL of each leaf sample was added with 3mL of the substrate solution and 1mL of pancreatic lipase assay. 6mL of tris-HCL buffer was added to the mixture. Then, the reaction of hydrolytic was done in water bath at temperature of 37ºC for about 30 minutes. Then, the 1 mL of stop solution was added which was a mixture of acetone and ethanol at 1:1 to stop the enzymatic activity. After that, the reading of absorbance was determined by using a spectrophotometer at wavelength of 410 nm. The results on inhibition were compared with standard activity (without inhibitor) in order to determine the percentage of inhibition. Gallic acid and quercatin were used as the control in this pancreatic lipase inhibition experiment. The step of the hydrolytic reaction was repeated for the reaction time of 40 minutes and 60 minutes for each sample. The enzymatic activity and the percentage of inhibition were calculated by using equation below.

III. RESULTS AND DISCUSSIONS

A. Determination of Particle size distribution by Mastersizer 2000E

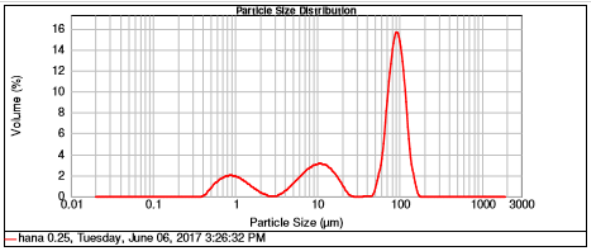

Figure 1: Graph of volume vs particle size

Figure above shows particle-size distribution vs the percentage of particle volume with different increasing ultrasonic pretreatment time using Mastersizer 2000E.

Based on the experimental results, the results is tabulated as follows:

I. Analysis of *Aquilaria Subintegra*

a) Data for 0.25µm *Aquilaria Subintegra* at different time

Table 1: Data for 0.25µm *Aquilaria Subintegra* at different ultrasonic reaction time

Time (min) / Descript.	30	60	90	120	150
Weighted residual (%)	6.199	11.808	10.869	7.234	4.319
Specific surface area m^2/g	1.16	0.722	0.0713	0.408	0.00521
d(0.1), µm	1.184	0.213	34.714	799.204	870.203
d(0.5), µm	73.632	13.096	1085.098	1195..426	1199.068
d(0.9), µm	1113.635	1149.612	1582.542	1577.275	1572.897

b) Data for 0.50 µm Aquilaria Subintegra at different time

Table 2: Data for 0.50 µm Aquilaria Subintegra at different ultrasonic reaction time

Time (min) / Descript	30	60	90	120	150
Weight residual (%)	4.957	6.405	12.74	5.007	6.162
Specific surface	0.0057	0.012	0.016	0.0183	0.0191
area m^2/g					
d(0.1), µm	753.528	828.336	635.103	994.567	662.533
d(0.5), µm	1108.993	1218.126	937.239	1266.031	1179.292
d(0.9)	1559.35	1421.902	1371.418	1115.648	1022.193

c) Data for 1.0 µm *Aquilaria Subintegra* at different time

Table 3: Data for 1.0 µm *Aquilaria Subintegra* at different ultrasonic reaction time

Time (min) / Descript	30	60	90	120	150
Weighted residual (%)	10.929	10.246	7.270	7.773	6.248
Obscuraion	0.01	0.00	0.03	0.00	0.04
Concentration	0.0013	0.00	0.0007	0.00	0.0005
Specific surface area m^2/g	0.00629	0.00661	0.00623	0.00552	0.0735
d(0.1) µm	619.463	597.473	664.664	765.674	661.462
d(0.5) µm	1044.790	980.212	1216.696	1156.801	1133.169
d(0.9) µm	1610.506	1536.831	1572.915	1623.039	1563.938

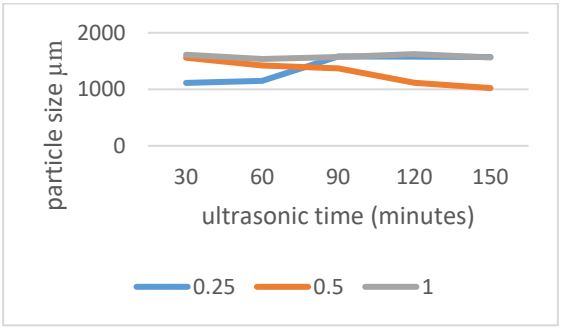

Figure 2: **Graph of particle size vs ultrasonic time of different grinded sizes of *Aquilaria Subtintegra* leaves**

Under this experimental condition, based on the result obtained, for *A.Subintegra*, for this grinded matured leaves of 0.25 mm, as the pretreatment time increased, the particle sizes are increased from 1113.635 µm at 30 minutes of pretreatment time to 1149.612 µm at 60 minutes and further increasing to 1582.542as ultrasonic time reach 90 minutes. For the next 120 and 150 minutes, the particle size decreases to 1577.275 and 1572.897 respectively. For 0.5 mm grinded leaves size, the particle sizes shows a fixed treand, which is at 30 minutes of ultrasonic time, the highest content particle sizes are at

1559.345 µm, while for 60 minutes of pretreatment the highest content of particle sizes are decreasing to 1421.902 µm, and as the ultrasonic pretreatment increased to 90 minutes, the particles decreased to 1371.418 µm, for 120 minutes and 150 minutes, the sizes increased to 1115.648 µm and 1022.193 µm respectively.

The other size of grinded dried A.Subintegra that has been use is 1.0 mm. From this experimental result, for the pretreatment time of 30 minutes, particle sizes are at 1610.506 and decreasing to 1536.831 µm at the pretreatment time of 60 minutes. For the next 90 and 120 minutes, the sizes are slightly increased to 1572.915 µm and 1623.039 µm respectively. At pretreatment time of 150 minutes, the sizes are decreased to 1563.938 µm.

II. Analysis of *Aquilaria Malaccensis*

a) Data for 0.25µm *Aquilaria Malaccensis* at different time

Table 4: Data for 0.25µm *Aquilaria Malaccensis* at different ultrasonic reaction time

Time (min) / Descrip	30	60	90	120	150
Weighted residual (%)	6.979	5.216	2.232	4.697	18.713
Uniformity	0.486	0.247	0.254	0.341	0.3
Specific surface area m^2/g	0.0275	0.0274	0.0326	0.0706	0.0851
d(0.1) µm	37.312	698.885	664.664	37.662	621.461
d(0.5) µm	974.400	1226.542	1216.696	1088.918	1168.057
d(0.9) µm	1692.092	1601.178	1599.915	1509.727	1402.22

a) Data for 0.5µm *Aquilaria Malaccensis* at different ultrasonic reaction time

Table 5: Data for 0.5µm *Aquilaria Malaccensis* at different time

Time (min) / Desc.	30	60	90	120	150
Weight Residual (%)	4.324	6.107	9.087	12.071	11.224
Specific surface area m^2/g	0.149	0.0348	0.0476	0.171	0.555
d(0.1)	14.289	114.122	897.013	15.442	5.032
d(0.5)	674.979	1057.999	1232.913	128.839	21.169
d(0.9)	1430.116	1467.170	1215.410	219.078	73.063

b) Data for 1.0 µm *Aquilaria Malaccensis* at different ultrasonic reaction time

Table 6: Data for 1.0 µm *Aquilaria Malaccensis* at different time

Time (min) / Desc.	30	60	90	120	150
Weight residual (%)	13.152	18.365	16.629	7.938	12.791
Obscuration	0.01	0.01	0.02	0.01	0.05
Concentration	0.00	0.0002	0.00	0.00	0.0001
Specific surface area m^2/g	0.0218	0.0348	0.289	0.363	0.916
d(0.1)	1.508	114.122	1.694	8.387	6.133
d(0.5)	72.930	1057.999	67.83	56.881	123.051
d(0.9)	1524.596	1467.170	209.042	117.058	114.36

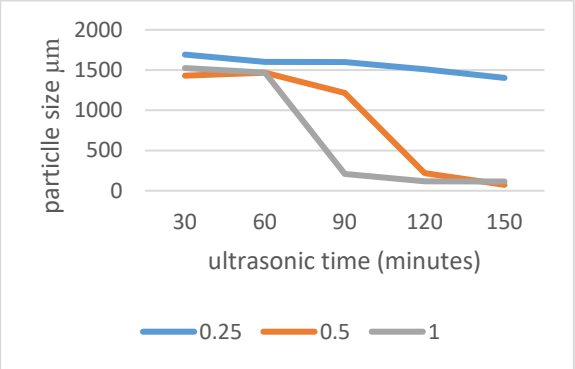

Figure 3: Graph of particle size vs ultrasonic time of different grinded sizes of *Aquilaria Malaccensis* leaves

This experiment is also tested to *A.Malaccensis* species with the ultrasonic pretreatment time as the function. For grinded A.Malaccensis leaves of 0.25 mm, the result obtained for determination of particle sizes does not shows a fixed trend as well, the result fluctuated across the pretreatment time. At 30 minutes the particle sizes are at 1692.092 µm, for 60 minutes the sizes then decreased to 1601.178 µm, the data obtained then further decreased to 1599.91 µm and 1509.727 at the time of 90 and 120 minutes, respectively. For 150 minutes, the sizes decreased to 1402.22 µm.

For 0.5 mm grinded leaves size, the particle sizes does not shows a fixed trend as well, which is at 30 minutes of ultrasonic time, the highest content particle sizes are at 1430.116 µm, while for 60 minutes of pretreatment the highest content of particle sizes are increasing to 1467.170 µm, and further increased as the ultrasonic pretreatment increased to 90 minutes, the particles decreased to 1215.410 µm, for 120 minutes and 150 minutes, the sizes decreased to 219.078 µm and further decreased to 73.063 µm. Another size of grinded dried *A.Malaccensis* that has been use is 1.0 mm. From this experimental result, the result obtained are fluctuated as well. For the pretreatment time of 30 minutes, particle sizes are at 1524.596 µm and increasing to 1467.170 µm at the pretreatment time of 60 minutes. For the next 90 and 120 minutes, the sizes are sudden decreased to 209.042 µm and slightly further decreased to 117.058 µm at 120 minutes.. At pretreatment time of 150 minutes, the sizes are decreased to114.36 µm.

Based on the trend that can be seen, there are only a few reading which are slightly different to expected result. However, most of the reading based on these two species indicate that as the ultrasonic reaction time increased, the particle sizes are reduced.

Theoretically, this decreasing of particle size was resulted by cavitation energy generated by ultrasonic. The cavitation energy produced shock waves that raised local pressure changed and shifted in liquid so that resulting in damaged on particle. Accordingly, it generated fragments of the particles as well as erosion on the surface so that resulted in particle diameter reduction. These data also support the morphology analysis in which destruction of particles increased when ultrasonic reaction was performed at the longer time and leading to a smaller particle size.

B. Interpretation of Pancreatic Lipase Inhibition Activity by using UV spectrophotometer

The inhibition of porcine pancreatic lipase by the leaf extracts were observed by the reduction of its activity in hydrolysis of p-NPP which releases p-nitrophenol. The pretreatment employed in the process of obtaining the extract was done to enhance the phenolic content of the extracts as these compounds have been found to be the major inhibitor of lipase enzyme.

Table 7: Different size and reaction time affecting percentage of lipase inhibition

Sample	Size, mm	Reaction time (minutes)	Absorbance (410 nm)	Percentage Inhibition (%)
Aquilaria Subintegra	0.25	30	0.029	51.67
		60	0.028	53.33
		90	0.025	58.33
		120	0.026	56.67
		150	0.029	51.67
	0.50	30	0.028	53.33
		60	0.027	55.50
		90	0.025	58.33
		120	0.028	53.33
		150	0.029	51.67
	1.0	30	0.026	56.67
		60	0.027	55.50
		90	0.029	51.67
		120	0.026	56.67
		150	0.028	53.33

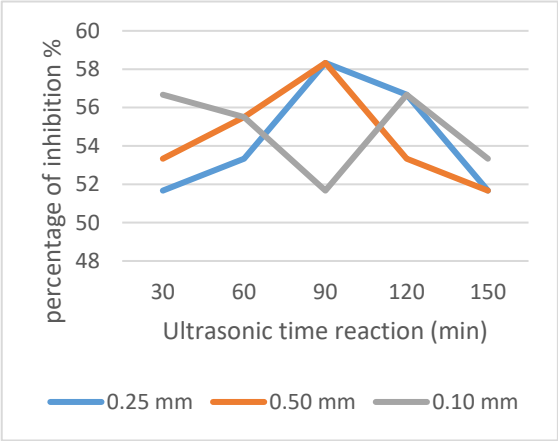

Figure 4: Graph of percentage of inhibition againts ultrasonic time reaction of different size of grinded *Aquilaria Subintegra* leaves

Based on the result obtained, for ***Aquilaria Subintegra as*** shown in Table 7, for 0.25 mm grinding size indicates that the optimum ultrasonic time reaction is at the time of 90 minutes ultrasonic time with the percentage of inhibition of 58.33%. For 0.50 mm grinding size of leaves, it also shows that 90 minutes, has the highest percentage of inhibition, at 58.33% as well. For 1.0 mm leaves size, that at 120 minutes, the percentage of inhibition is at the highest, which is 56.67%. From table 8, shows the inhibition activity across the increasing time of ultrasonic reaction for 30 to 150 minutes of 3 different sizes of grinded *Aquilaria Subintegra* leaves.

Table 8: Different size and reaction time affecting percentage of lipase inhibition

Sample	Size, mm	Reaction time (minutes)	Absorbance (410 nm)	Percentage Inhibition (%)
	0.25	30	0.031	48.33

Aquilaria Malaccensis		60	0.031	48.33
		90	0.029	51.67
		120	0.030	50.00
		150	0.032	47.00
	0.50	30	0.032	47.00
		60	0.030	50.00
		90	0.029	51.67
		120	0.029	51.67
		150	0.031	48.33
	1.0	30	0.033	45.00
		60	0.033	45.00
		90	0.031	48.33
		120	0.031	48.33
		150	0.030	50.00

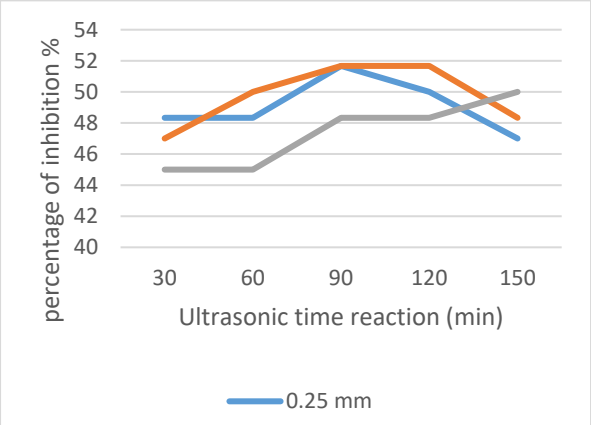

Figure 5: Graph of percentage of inhibition against ultrasonic time reaction of different size of grinded *Aquilaria* Malaccensis leaves

In this research, Aquilaria Malaccensis is also studied at 3 different leaves sizes, 0.25,0.50 and 1.0 mm. For 0.25 mm grinded leaves, at 90 minutes shows the highest percentage of inhibition with the percentage of inhibition og 51.67%. 0.50 mm of leaves size has the highest percentage of 51.67% at the ultrasonic time of 90 and 120 minutes. It indicated as the range time from 90 to 120 minutes, the inhibition activity of phenolic compounds does not change. At 1.0 mm leaves size, at the time of 150 minutes, inhibition activities shows the highest activities, which inhibits about 48.33%. From **the** Figure 5, shows the inhibition activity across the increasing time of ultrasonic reaction for 30 to 150 minutes of 3 different sizes of grinded *Aquilaria Malaccensis* leaves.

The result obtained for both species indicate that the inhibition activity is at the highest on the ultrasonic time of 90 minutes. Ultrasonication of these samples were done at the temperature of 60 ºC since studies shows that 60 ºC is the optimum temperature of recovery these phenolic cimpound.

These research has revealed that the ultrasonic reaction time does effect the particle size. As the ultrasonic reaction time increases, the particle size reduced. The determination of inhibitory activity of phenolic compound in this study has found that the optimum time for ultrasonic reaction is at 90 minutes with the temperature of 60 ºC. These can be concluded that particle sizes does effecting the inhibitory activity of phenolic compounds in the leaves.

As the particles size or volume decreased, the surface area of particles increases, thus wider the efficiency of particle in inhibitory activities. However, by prolonging the time of ultrasonic reaction at 60 ºC, which is quite high temperature, will not increase the efficiency of inhibitory activity but lower the activity. This is because, by increasing ultrasonic time at high temperature will only increase the chance of oxidation of phenolic compound, thus resulting in the lower of the activity of anti-obesity properties (Zaki, 2015)

CONCLUSIONS

Ultrasonic damages and fragments the particles. This can be shows by the reduction of particle sizes as the reaction using ultrafiltration time increased. In inhibitory activity of phenolic compound in these two species of *Aquilaria Subintegra* and *Aquilaria* Malaccenis, at the optimum temperature of 60 ºC, the inhibition of pancreatic lipase increases as time increase as well. However this activity is lower as the ultrasonic time reaction exceeding 90 minutes.

REFERRENCES

[1] W.-K. Chen, (1999). *Linear Networks and Systems* . Belmont, CA: Wadsworth. 123–135.
[2] H. Poor, (2013). *An Introduction to Signal Detection and Estimation*. Chapter 4. New York: Springer-Verlag.
[3] Smith (2008). An approach to Graphs of Linear Form. Unpublished.
[4] R. J. Vidmar, (1992). On the Use of Atmospheric Plasmas as Electromagnetic Reflectors. *IEEE Trans. Plasma Sci. 21(3).* 876-888.
[5] Afiffudden, S. K. N., Alwi, H., & Hamid, K. H. K. (2015). Determination of 4'-Hydroxyacetanilide in Leaves Extract of Aquilaria malaccencis by High Pressure Liquid Chromatograph. Procedia - Social and Behavioral Sciences, 195, 2726–2733. http://doi.org/10.1016/j.sbspro.2015.06.381
[6] Chemistry Letters, 23(12), 3604–8. http://doi.org/10.1016/j.bmcl.2013.04.013
[7] Ballinger, A., & Peikin, S. R. (2002). Orlistat: its current status as an anti-obesity drug. European Journal of Pharmacology, 440(2-3),

109–117. http://doi.org/10.1016/S0014-2999(02)01422-X

[8] Helen, L. Y. S., Rahim, A. A., Saad, B., Saleh, M. I., & Raja, P. B. (2014). Aquilaria Crassna Leaves Extracts – a Green Corrosion Inhibitor for Mild Steel in 1 M HCl Medium, 9, 830–846.

[9] Ibrahim, a. H., Al-Rawi, S. S., Majid, a. M. S. A., Rahman, N. N. A., Salah, K. M. A.-, & Kadir, M. O. A. (2011). Separation and Fractionation of Aquilaria Malaccensis Oil Using Supercritical Fluid Extraction and tThe Cytotoxic Properties of the Extracted Oil. Procedia Food Science, 1, 1953–1959. http://doi.org/10.1016/j.profoo.2011.09.287

[10] Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. Food Chemistry, 126(4), 1821–1835. http://doi.org/10.1016/j.foodchem.2010.12.026

[11] Jamahseri, N. F., Najwa, M., Rodhi, M., Zulkarnain, N. H., & Husain, N. C. (2014). FTIR Analysis of Phenolic Compound as Pancreatic Lipase Inhibitor from Inoculated Aquilaria Malaccensis. The Malaysian Journal of Analytical Sciences, 18(3), 683–689.

[12] Nivas, D. (n.d.). Phytochemical screening and in-vitro antioxidant activities of Colubrina asiatica Brong Retrieved from http://www.jocpr.com/articles/phytochemical-screening-and-invitro-antioxidant-activities-ofcolubrina-asiatica-brong.pdf