AN INTEGRATED ULTRASONIC-ASSISTED ENZYMES AS A SINGLE EXTRACTION UNIT FOR GALLIC ACID FROM *LABISIA PUMILA* (KACIP FATIMAH)

NOOR ADILAH BINTI MD SALEHAN

Master of Engineering (Chemical)

UNIVERSITI MALAYSIA PAHANG

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900702-01-5842	Dr. Azilah binti Ajit@Abd Aziz
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_____

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Full Name : DR. AZILAH BINTI AJIT @ ABD AZIZ

Position : SENIOR LECTURER

Date :

_____

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ABSTRAK

Ekstrak *Labisia pumila* (di sini dikenali sebagai Kacip fatimah) mengandungi sebatian fenolik seperti asid galik yang mempunyai pelbagai kesan biologi. Pengekstrakan terbantu ultrabunyi (ultrasound-assisted extraction, UAE) dilaporkan secara meluas untuk pengekstrakan tumbuhan ubatan dan herba kerana teknologinya yang menjimatkan dan hijau. Oleh itu, kajian ini menggunakan UAE berserta pengekstrakan enzim (enzymatic extraction, EnE) bagi meningkatkan hasil pengekstrakan asid galik daripada *Labisia pumila*. Pengaruh lima parameter terhadap pengekstrakan asid galik daripada *Labisia pumila* dikaji: masa pengekstrakan (1–8 h), nisbah sampel kepada pelarut (1:6, 1:8, 1:10, dan 1:12 berat/berat atau wt/wt), suhu (40, 50, 60, dan 80 °C), ultrasonik (10%, 20%, dan 40% kitar tugas), dan kepekatan selulase (0.025, 0.05, 0.1, 0.2, dan 0.3 g/L). Keamatan kuasa pada 8.66 W/cm2 dikenakan menggunakan pemproses ultrasonik Q700 (700 watts, 20 kHz). Keputusan menunjukkan bahawa hasil asid galik adalah yang tertinggi pada 50 °C dengan nisbah sampel kepada pelarut 1:10 melalui pengekstrakan akueus (aqueous extraction, AE) yang menghasilkan 1.0251 ± 0.0569 mg/g asid galik. Manakala, teknik UAE dan EnE memberikan hasil tertinggi pada kitar tugas 40% dan kepekatan selulase 0.05 g/L iaitu masing-masing 1.8425 ± 0.1191 mg/g and 1.28565 ± 0.1760 mg/g asid galik. Keputusan pengekstrakan enzim terbantu ultrabunyi (ultrasound-assisted enzymatic extraction, UAEnE) menunjukkan pola peningkatan apabila tempoh pengekstrakan ditambah daripada 1 kepada 7 jam. Walau bagaimanapun, bagi tempoh melebihi 7 jam, hasil pengekstrakan berkurangan yang menunjukkan bahawa degradasi asid galik mungkin telah berlaku. Jumlah asid galik tertinggi pada 2.9287 ± 0.4060 mg/g diperoleh melalui UAEnE selepas 7 jam pengekstrakan dengan kenaikan sebanyak 2.91 kali berbanding dengan keputusan AE. Dengan itu, sebuah unit bersepadu pengekstrakan enzim terbantu ultrabunyi berjaya menambah baik pengekstrakan asid galik daripada *Labisia pumila*.

ABSTRACT

Labisia pumila (locally known as Kacip fatimah) extract contains phenolic compounds such as gallic acid that have multiple biological effects. Ultrasound-assisted extraction (UAE) is widely reported for the extraction of medicinal plants and herbs due to its economical and green technology. Therefore, this study applied UAE with unified enzymatic extraction (EnE) to enhance the extraction yield of gallic acid from *Labisia pumila*. The influence of five parameters on the extraction of gallic acid from *Labisia pumila* were investigated: extraction time (1–8 h), sample-to-solvent ratio (1:6, 1:8, 1:10, and 1:12 wt/wt), temperature (40, 50, 60, and 80 °C), sonication (10%, 20%, and 40% duty cycle), and cellulase concentration (0.025, 0.05, 0.1, 0.2, and 0.3 g/L). The power intensity of 8.66 W/cm2 was implemented using an ultrasonic processor Q700 (700 watts, 20 kHz). The results showed that the gallic acid yield was the highest at 50 °C with 1:10 sample-to-solvent ratio for aqueous extraction (AE) with 1.0251 ± 0.0569 mg/g of gallic acid extracted. Whereas, both ultrasound-assisted extraction (UAE) and enzymatic extraction (EnE) techniques gave the highest yield at 40% duty cycle and 0.05 g/L cellulase concentration with 1.8425 ± 0.1191 mg/g and 1.28565 ± 0.1760 mg/g gallic acid extracted, respectively. The result of the ultrasound-assisted enzymatic extraction (UEnE) indicated an increased trend when the extraction time was increased from 1 to 7 h. However, beyond 7 h the yield declined indicating that the degradation of gallic acid may have initiated. The highest gallic acid amount obtained from UAEnE was 2.9287 ± 0.4060 mg/g after 7 h of extraction with 2.91-fold increment compared with the AE result. Thus, the integrated single unit ultrasonic-assisted enzyme extraction successfully improved the gallic acid extraction from *Labisia pumila*.

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LIST OF SYMBOLS

kHz	Kilo hertz
wt.%	Weight percent
µg/ml	Microgram per milligram
mg/ml	Milligram per gram
°C	Degree Celsius
h	Hour
mm	Millimeter
%	Percent
W	Watt
min	Minutes
rpm	Revolution per minute
nm	Nanometer
s	Second
W/cm^2	Watt per centimeter square
units/g	Unit per gram
M	Molar
g/L	Gram per liter
µm	Micrometer
mg/L	Milligram per liter
mg	Milligram
kg	Kilogram
l	Volume of solvent during extraction
ppm	Part per million
g/ml	Gram per milliliter
mg/g	Milligram per gram
fold	Fold
m/z	Mass-to-charge ratio

LIST OF ABBREVIATIONS

AE	Aqueous extraction
EnE	Enzymatic extraction
FESEM	Field Emission Scanning Electron Microscope
HPLC	High performance Liquid Chromatography
HPLC-DAD	High performance Liquid Chromatography Diode-Array Detection
LCMS-Q-TOF	Liquid Chromatograph Mass Spectrometer Quadrupole Time-of-Flight
PMN	Plant metabolite network
tR	Retention time
UAE	Ultrasound-assisted extraction
UAEnE	Ultrasound-assisted enzymatic extraction
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background

The scientific history of ultrasound is rooted from the study of sound waves theory by Sir Isaac Newton in 1687 (Mason, 1998). Sound waves are divided into three categories which are audible, infrasonic, and ultrasonic waves. Ultrasound has high-frequency sound waves (>20 kHz) which is out of human hearing limits. Ultrasound has been extensively used in the fields of medicine, pharmaceutical, food technology, and analytical chemistry. Besides that, it has been used in many industries for cleaning and mixing, and to enhance chemical processes. Ultrasound-assisted extraction (UAE) has been recognised for its potential industrial application in the phytopharmaceutical extraction industry for a wide range of herbal extracts. UAE can potentially improve extraction process since it enhances diffusive transport by better mixing and is able to loose the bond present in the sample complex matrix.

Ultrasonication generates alternatively low- and high-pressure waves in the liquid which leads to the formation and violent collapse of microbubbles. This phenomenon creates cavitation in the liquid. The cavitation caused by the microbubbles produces high-speed liquid jet and strong dynamic shear force. Ultrasonic cavitation improves the mechanical effect of substrate and bond breaking (Chen et al., 2011). The intracellular products are easier to release when the bond is broken down by sonication. UAE causes the split of the αether linkages between lignin and hemicelluloses chains and therefore improves the extraction with respect to yield and purity (Hollmann et al., 2009). Ultrasound has been used to increase the extraction efficiency of the herbs (Albu et al., 2004) and enhance the existing extraction processes enabling new commercial extraction opportunities and processes (Vilkhu et al., 2008). UAE method increases the

yield, enhances the rate of extraction, shortens the extraction time, and lowers the extraction temperature (Yang et al., 2013). Referring to previous studies, low intensity of sonication is enough to enhance the extraction process. At the power intensity of 1 W/cm2, ultrasound gives a higher yield in the extraction of antioxidants from Rosmarinus officinalis (Toma et al., 2001). It can be concluded that ultrasound improves the permeability of the cell wall, mechanical stressing, and cavitation effect during the extraction process.

Complex matrix in plant cell wall is the major obstruction to extract active compounds from the plant and one of the ideas to overcome it is by using cellulase enzyme to hydrolyse cellulose and hemicellulose that are the most abundant polysaccharide components in the cell wall (Pauly & Keegstra, 2008). To improve the accessibility of the target compounds during aqueous extraction processes, the complex matrix needs to be loosened. This biotechnological procedure has successfully improved the extraction of important metabolites from seaweeds (Wijesinghe & Jeon, 2012), Ginkgo biloba leaves (Chen et al., 2011), and coconut (Man et al., 1996).

The application of ultrasound along with enzymes enhances various biochemical activities such as enzymatic bioactive metabolites extraction (Tiwari, 2015), extraction of polysaccharides from Epimedium leaves (Chen et al., 2012), enzymatic hydrolysis of cellulose (Sulaiman et al., 2013), and fermentation processes (Sulaiman et al., 2011). Ultrasound enhances the enzyme activity by improving the collision between enzyme and its substrates which results in higher reaction rate. Furthermore, ultrasonication can help splitting the α-ether linkages between lignin and hemicelluloses chains thus improving the extraction with respect to yield and purity (Hollmann et al., 2009). The application of ultrasound in the extraction of *Labisia pumila* is expected to increase the production of a marker compound, gallic acid, without damaging the properties of this active compound since only mild temperature is involved.

1.2 Problem statement

Herbal-based products are getting a widespread acceptance among consumers and being the preferred alternative medicine. The increasing demand of herbal-based product is most probably due to the awareness to use natural and safe product as supplement and alternative to medicine to maintain health and treat illness. Herbs not only help in sustaining a healthy life, but also can be used for treating chronic diseases

(Verma & Singh, 2008; Ved & Goraya, 2007). Conventional and many previous works on extraction usually will take a long period with high temperature. Besides, it also involves chemicals such as solvent throughout the extraction process. The addition of enzyme in the extraction method is proven to enhance the releasing of intracellular compound and sonication also improves the accesibility of the active compund by the cavitation power. To fill the gap from the previous work of extraction, the identification of the best method to produce safe product needs to be done. In this research, integrated ultrasonic-assisted enzymes as a single extraction unit for gallic acid from *Labisia pumila* (Kacip fatimah) is applied. This is an appropriate method to improve the quantity and quality of the extraction yield without involving any hazardous chemical which has not been reported yet.

Lignocellulosic biomass in *Labisia pumila* contains a large amount of valuable bioactive compounds for the applications in food, nutraceutical, cosmeceutical, and pharmaceutical industries. However, they are covalently bonded with lignin and other carbohydrates within the complex matrix in the plant cell wall, thus restricting them from undergoing any chemical changes and enzymatic degradation. Hence, conventional extraction of herbs is a time-consuming process. Therefore, the knowledge in ultrasound-assisted extraction is needed to enhance the yield and speed up the process of fine herbal extract to break the bond then expose the components and improve the intracellular compound released (Galvan et al., 2012).

Many studies have been done using ultrasound to enhance extraction yield and enzymatic extraction (Sindhu et al., 2013; Li et al., 2004). However, no studies were done on the combination of ultrasound with enzymes for enhancing the extraction of gallic acid from *Labisia pumila*. The aim of this study was to investigate the effect of ultrasound on the enzymatic extraction of gallic acid from *Labisia pumila*.

1.3 Objectives

This work focused on examining the effects of ultrasound on the extraction of *Labisia pumila*. Sonication regimens which could influence a process relative to control were identified. Attempts were made to understand the possible causes of ultrasound

induced enhancement in the diverse model of extraction situations. The objectives of this work were:

i. To investigate the performance of the new method integrated ultrasonic-assisted enzyme extraction as a single extraction unit for gallic acid from *Labisia pumila* (Kacip fatimah)
ii. To compare the performance of the proposed new method integrated ultrasonic-assisted enzyme extraction (UAEnE) with aqueous extraction (AE), ultrasound-assisted extraction (UAE), and enzymatic extraction (EnE)
iii. To characterise and analyse the produced gallic acid extracted from *Labisia pumila* (Kacip fatimah)

1.4 Scope of the research

To achieve the objective, scopes were identified in this research. The scopes of this research were:

i. Characterise the conventional extraction for water-based systems (control) in terms of temperature (40–80 °C), sample-to-solventratio (1:6, 1:8, 1:10, and 1:2), and extraction period to achieve maximum yield.
ii. Examine the best condition of sonication regiments with difference duty cycle levels (10%, 20%, and 40%) at low power intensity.
iii. Examine the best enzyme concentration (0.025, 0.05, 0.1, 0.2, and 0.3 g/L) for the extraction of gallic acid from *Labisia pumila.*
iv. Investigate the performance of unified analysis between ultrasound and enzymes for novel process of *Labisia pumila* extraction
v. Analyse gallic acid production using high performance liquid chromatography coupled with diode array detector (HPLC–DAD), liquid chromatography mass spectrophotometer-quadrupole time-of-flight (LCMS–QTOF), and morphology study using field emission scanning electron microscopy (FESEM).

1.5 Significance of study

At present, extraction of herbs generally involves a solvent such as hexane. However, the main concern of this process has been the safety implications to the

surrounding. This study provides a useful contribution to the extraction process of herbs especially *Labisia pumila*. This study can improve the yield of extraction successfully. The absence of established procedure for the herbs extraction consumes time and energy and gives negative impact to the surrounding. Perhaps this study can be the first step to introduce the more effective method to extract active compounds from herbs.

This study used an aqueous extraction as the base line to make easier to see the improvement of each method involved in the extraction in reference to the amount of gallic acid yield extracted in each sample. All of the three methods tested in this study improved the gallic acid yield. However, the integrated ultrasound-assisted enzymatic extraction performed the best.

CHAPTER 2

LITERATURE REVIEW

The growth and development in herbal research based products are getting wide spread acceptance among consumers and being the preferred alternative medicine. Herbal not only helps in sustaining a healthy life, it also can be used as alternative treatment for chronic diseases. Malaysian women traditionally use *Labisia pumila* during childbirth to induce and ease the delivery process. It is also used as a post-partum medication to help contract the birth channel, regain body strength, regulate menstrual cycle and avoid painful menstruations. In addition, *Labisia pumila* is also used to relieve the menopausal symptoms (Chua et al., 2012).

2.1 *Labisia pumila*

Labisia pumila (Figure 2.1) is a species of small woody and leafy plants belonging to the Myrsinaceae family that can widely be found in the tropical forest of South East Asian countries (Chua et al., 2012). It has creeping stems and is mainly found in the lowland and hill forests in Southeast Asia, particularly Malaysia, Indonesia, Thailand, Laos, Cambodia, and Vietnam (Farouk et al., 2008) and mostly obtained from the natural tropical forest (Fazwa et al., 2013). It can be recognized as a small herbaceous under a shrub that roots from the stem with a few leaves pointing upwards with the spike like panicle of small clusters of white or pink flower (Pattiram et al., 2011). There are eight varieties of *Labisia pumila* (Sunarno, 2005), but only three of the varieties are widely found and studied; *Labisia pumila* var. pumila, var. alata and var. Lanceolata (Chua et al., 2012). Varieties of *Labisia pumila* can be differentiated from each other by their petiole and leaf characteristics. *Labisia pumila* var. Alata has a winged petiole and red veins, while var. pumila has a marginate petiole and ovate leaf blade shape, and var.lanceolata has a long and non-winged petiole. *Labisia pumila* var.

alata, most commonly encountered variety in Malaysia, is an important medicinal plant that is widely used as alternative medicine and supplements for women health and beauty. Besides that, *Labisia pumila* also has high potential in the management of chronic diseases (Nik Hussain & Kadir, 2013). Hence, *Labisia pumila* become the subject of intense pharmacology and chemical studies for recent years.

The uniqueness of *Labisia pumila* is the contain of phenol metabolites (Karimi & Jaafar, 2011) which can be used as a chemical marker. Based on previous studies, this metabolites has been proven to have multiple biological effects such as high antioxidant properties (Chua et al., 2011) and anti-inflammatory activity (Vijayalakshmi & Ravindhran, 2012). The main function of antioxidants is to delay the oxidation processes of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals, this could at least in part be due to the presence of one of the important phytochemical, gallic acid (3,4,5-trihydroxybenzoic acid). The antioxidant activity of benzoic acids has been reported higher than vitamin C and E against reactive oxygen species (Chua et al., 2012). The previous studies on the extraction of *Labisia pumila* are summarized in Table 2.1.

Figure 2.1 *Labisia pumila* var alata

Source: Chua et al. (2012).

Table 2.1 Extraction of phytochemical from *Labisia pumila*

***Labisia pumila* species**	**Active Compound Tested**	**Condition**	**Findings**	**Reference(s)**
Not specified	Gallic acid	Temperature = 40 °C Sample-to-solvent Ratio = 1:10 Time = 4 h	13.42 wt.%	Mohd Azrie et al. (2014)
Var alata	3,4,5-trihydroxybenzoic acid.	Temperature =80 °C Sample-to-solvent Ratio = 1:6 Time =3 h	Not specified	Abdul et al. (2012)
Not specified	Antioxidant	Temperature =100 °C Sample-to- olvent Ratio = 1:10 Time =4 h	60.82 µg/mL	Choi et al. (2010)
Not specified	Not specified	Temperature =80 °C Sample-to-solvent Ratio = 1:10 Time =3 h	10-12 % yield	Zulkarnaini et al., (2013)
Var alata	Not specified	Temperature = 80°C Sample-to-solvent Ratio = 1: 6 (double stage) Time =3 h	Not specified	Al-Wahaibi et al., (2008)

2.2 Gallic acid

Gallic acid (3,4,5-trihdroxy-benzoic acid) is recommended marker component in extraction of *Labisia pumila* (Malaysian Standard, 2013). It can be present as free acid, bonded to hydrolyzable tannin, and also as intermolecular compound for ester and cyclic ether-ester. Gallic acid is a phenolic acid and phytonutrient which is characterized as a strong antioxidant. The mechanism of gallic acid and its derivatives has been reported in several studies such as its anticancer property (Maurya et al., 2011), food preservation (Jiao et al., 2014), and antimicrobial against human pathogens (Karamae et al., 2006). Karamae et al. (2006) proved that, among natural polyphenols, gallic acid is a successful model for the radical properties. Hence, for this study, gallic acid is selected as marker compound. The chemical formula of gallic acid is $C_7H_6O_5$ with a molecular mass of 170.12 g/mol. Their chemical structures are shown in Figure 2.2. The main function of antioxidants function is to delay the oxidation processes of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions

by free radicals.. Gallic acid and its derivatives are widely found in various plants and fruits (Wang et al., 2015 ; Chen et al., 2009).

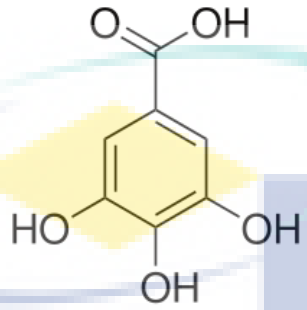


Figure 2.2 The chemical structures of gallic acid

Source: Aruoma (1993)

2.3 Extraction methods

Extraction is a process of separating desired components from a material. Vinatoru (2001) stated that herb extraction is defined as the recovery of the desired compounds or compound mixtures from fresh or dried plants. Increasing in the demand for the herbal extractions, conventional method requires continuous improvements and modifications. There are several conventional extraction methods such as infusion, decoctions, and maceration. However, the disadvantages of use of conventional method include longer time period and excess use of chemicals. For example, in the maceration extraction methods, the process takes 2 to 7 days with massive amount of solvent (Devgun et al., 2012). Non-conventional extraction methods include ultrasonically assisted, enzyme, and microwave assisted is efficient methods to improve the conventional extraction process (Puri et al., 2012; Cravotto et al., 2008). Water is selected as a solvent in the extraction process of gallic acid from *Labisia pumila* (Kacip fatimah). Refer to Yeop et al., 2017, using water as solvent to extract gallic acid from *Labisia pumila* (Kacip fatimah) yielded about 29% higher than ethanol for particle size ranged from 250-500 μm.

2.3.1 Aqueous extraction (AE)

Aqueous extraction is a conventional extraction method which is not involved or used any chemical. In this method, the sample will be heated for a certain time. However, since the process only depends on the heat supplied to extract the desired component, the process usually will take along period. This is time consuming. Indirectly it was also energy consuming because need to supply heat for a long period. In the other hand, this method was the most environmental friendly since it was not involved any chemical (Rosenthal et al., 1996).

Infusion and decoction are nearly similar extraction process. Both of them involve materials suspended in the water or solvent over the time depends on the characteristic of the desired compound. Water was categorized as GRAS (Generally Recognized as Safe) for extraction of any intracellular cell from plant (Monroy et al., 2016). Infusions usually use to extract vitamin or volatile ingredients from soft ingredient like leaves and flower. Whereas, decoction is used to extract the compound from hard materials such as root, bark and seed. Both methods are the simplest and easiest extraction method. For this method, the plant or herbs will be added to the boiled water. After certain time, the boiled plant is filtered and then the solvent used will be analysed. In maceration the materials is continuously extracted with a solvent at particular time and temperature using soxhlet apparatus.

2.3.2 Ultrasound-assisted extraction (UAE)

Ultrasound has been recognised for potential industrial application in the phyto-pharmaceutical extraction industry for a wide range of herbal extracts. Ultrasound-assisted extraction (UAE) process enhancement for food and allied industries include herbal, oil, protein and bioactive from plant and animal materials. UAE method is able to increase yield of extracted components, increase rate of extraction, and achieve reduction in extraction time and higher processing throughput (Vilkhu et Al.,2008). Vilkhu et al. (2008) reported that ultrasound can enhance existing extraction processes and enable new commercial extraction opportunities and processes. Figure 2.3 shows the mechanism on how ultrasounds induce the cell damage to release intracellular compounds.

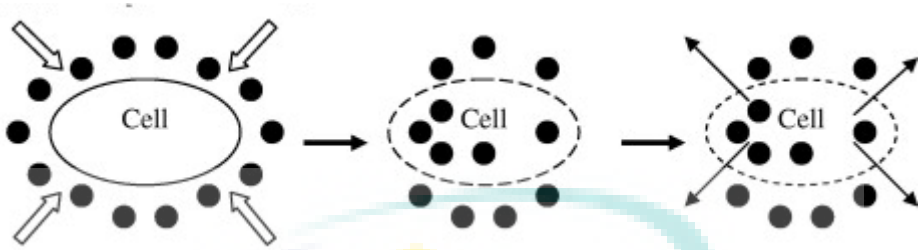


Figure 2.3 Mechanism of ultrasound-induced cell damage

Source: Chemat et al. (2011)

Living tissues where the desired components are localized in surface glands can be stimulated to release the components by relatively mild ultrasonic stressing (Toma et al., 2001). In tissues where the desired components are located within cells, pre-ultrasound treatment by size reduction to maximise surface area is critical for achieving rapid and complete extraction (Balachandran et al., 2006). Devgun et al. (2012) reported that ultrasonic-assisted extraction technique enables automation, shortened extraction time and reduce organic solvent consumption. The UAE performance is affected by the factors including intensity, time, solvent, temperature, pulsation and matrix. Besides that, UAE involve mechanical vibrations from high frequency sound waves. Ultrasound can increase in the permeability of the cell wall, mechanical stressing and cavitation effect during the extraction process (Suslick, 1989 ; Baker et al., 2001 ; Karshafian et al., 2009). During sonication, thousand of cavitation bubbles created from the tip rapid vibration. During these bubble explosions, it was releases tremendous energy in the cavitation field (Barati et. al., 2007).This contribute on enhancement of extraction process gallic acid from the *Labisia pumila* sample. Figure 2.4 shows how cavitation bubbles was destroy the cell wall matrix which result in emprovement in extracting intracellular compound include gallic acid from *Labisia pumila*. Sonication suitable for laboratory-scale and large-scale extraction because it easy to handle with short extraction time and high extract quality (Mohammad Azmin et al., 2016).

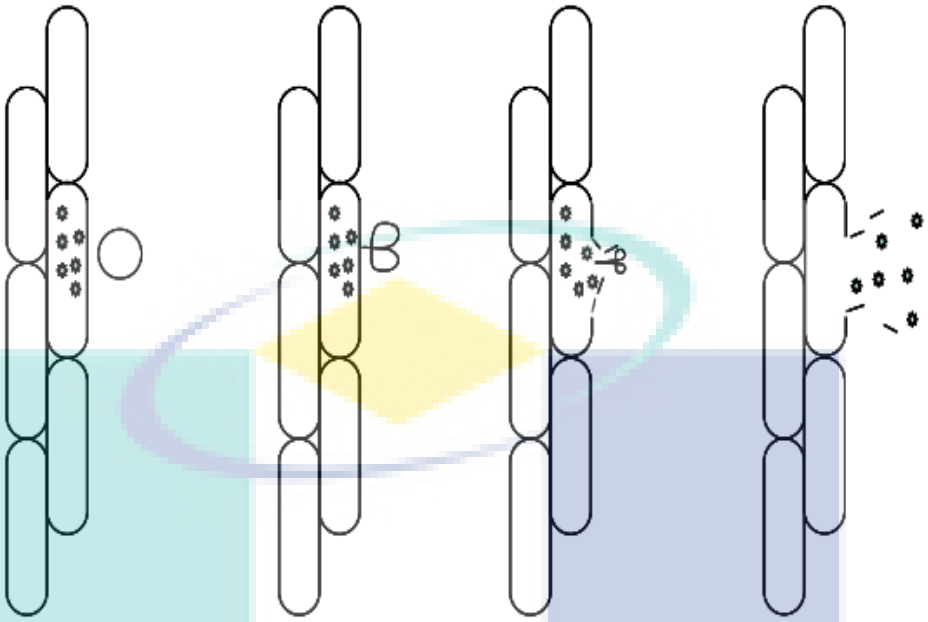


Figure 2.4 The mechanism of cavitation bubbles destroys the cell wall matrix. (a) The cavitation bubble formed by the sonication, (b) The formation of microjet result from compression of cavitation bubble, (c) Microjet attack the cell wall matrix with high pressure and temperature formed from the cavitation bubble collapsing, and (d) The intracellular compound was release through the attacked cell wall matrix

Source: Chemat et al. (2011)

2.3.3 Enzymatic extraction (EnE)

Degradation of cellulose involve 3 major classes of complex cellulase namely endocellulase, exocellulase and β-glucosidase or also known as cellobiase. Figure 2.5 shows the mechanism of cellulose degradation by three complex cellulase namely endocellulase, exocellulase and β-glucosidase or cellobiase. Firstly, endocellulase will attack the reactive region in the cellulose chain and produce free end chain. Then, exocellulose will further degrade the cellulose by removing cellobiose unit from the end chain. After that, to complete the hydrolysis of cellulose to glucose, cellobiase will convert detached cellobiose to glucose monomer. Hence, by hydrolysis of the cellulose in the cell wall will definitely facilitate extract release of important metabolites (Puri et al., 2012).

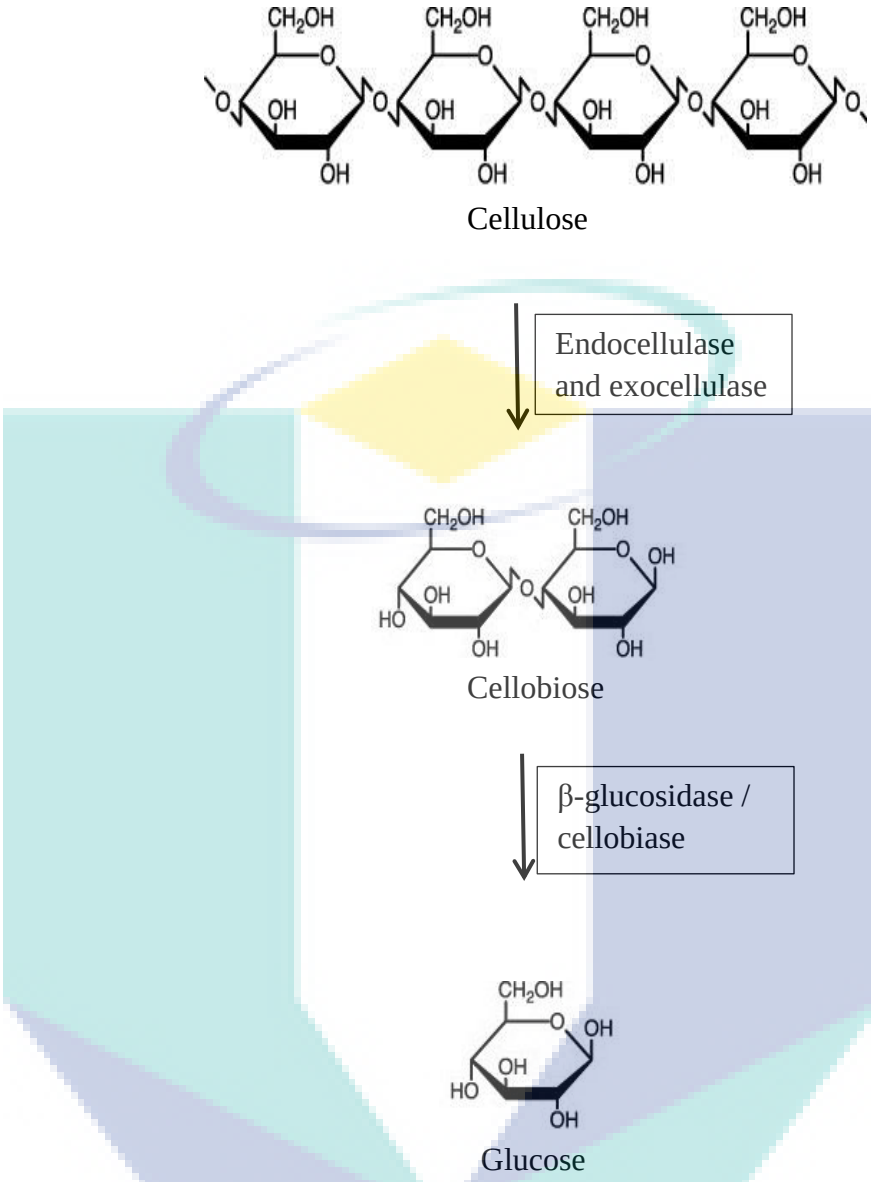


Figure 2.5 The mechanism of cellulose degradation by three complex cellulase namely endocellulase, exocellulase and β-glucosidase or cellobiase

Source: Yoshida et al. (2004)

Enzymatic pretreatment will facilitate cell matrix destruction. It can be a better method of choice to improve the extraction process (Gavit et al., 2011). From Gavit et al (2011) study found that the extraction of sugar from soyabean was increased from 2.78% to 4.46% with application of cellulase. Extraction process will be more effective without or less barrier to extract the desired component. Moreover, enzymatic hydrolysis were examined to enhance the efficiency of extraction, after the enzymatic hydrolysis, the extraction yield was improved from 1.29 to 1.73-fold (Cho et al., 2013). The enzymatic treatment may breakdown the protein networks or bonds at cotyledon

cells (Gai et al., 2013). The enzyme types, enzyme concentration and extraction time are important variables that can influence the extraction yield (Gai et al., 2013).

Accessibility of cellulose by cellulase was the major factor for digestibility of pretreated lignocellulosic substrates (Zhu et al., 2009). Lignins can reduce hydrolysis rates of cellulase by acting as a physical barrier, binding with the enzymes and inhibiting the reaction of enzyme (Pan, 2008). The pretreatment step is needed to enhance the accessibility to enzymes. Steam explosion has proven to be efficient pretreatment to improve the reaction of enzyme towards lignocellulosic substrates (Kumar et al., 2010). Appropriate pretreatments need to be developed for efficient degradation and enzymes reaction. Enzyme adsorption to cellulose might be a determining factor for efficient hydrolysis to occur (Heiss et al., 2011).

2.3.4 Ultrasound-assisted enzymatic extraction (UAEnE)

Sonication can promote enzyme reaction by facilitating swelling, enlarged pores of the cell wall and hydration. Enlarging pores of the cell wall phenomenon enhance diffusion, reaction and mass transfer. It can facilitate both of the enzyme reaction and the extraction process. Hence, the extraction of alkaloids from cinchona bark was increased by 15% with the application of ultrasound compared to soxhlet extraction (Vinatoru, 2001). Wang et al., (2012) reported that the cellulase activity rate can be improved with low-intensity ultrasound at 60 W, 24 kHz for 10 min. The enzyme activity was increased by 24.67% over the control. Cavitation is the formation, growth and collapse of vapour or gas bubbles that occur with ultrasound which will give direct effect on the enzyme molecules and enhances the mass transfer in the heterogeneous processes (Weavers et al., 1998).

The ultrasound modification on enzyme activity can promote the enzyme activity with having far-reaching consequences. Changes in activity of the enzyme in the ultrasonic bath can be influenced by the parameters of the sonication and the characteristics of the enzyme. Thus, the enzyme macromolecule interaction has a significant effect on the efficiency of the bioprocess. Much study has been done stated that the overall effect of the ultrasound is very positive but certain enzyme can be

sensitive to ultrasound irradiation in particular cases, for example, the ultrasound can damage the enzyme (Szabo & Csiszar, 2013).

2.4 Analysis Method

Three methods were used for qualitative and quantitative analysis for this research. Firstly, the extracted gallic acid concentration was analysed using high performance liquid chromatography (HPLC). Then, for qualitative analysis, liquid chromatography mass spectrophotometer-quadrupole time-of-flight (LCMS-QTOF) at was done. Other than that, an extracted leaves was analysed by using field emission scanning electron microscopy (FESEM) to study the effect of each extraction method towards plant leaves structure.

2.4.1 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) was used for quantitative analysis. Figure 2.6 shows the high-performance liquid chromatography (HPLC) Systems which have six main parts namely as solvent/mobile phase reservoir, pump, auto sampler injector, column, detector and computer. A reservoir holds the mobile phase and sufficient mobile phase for each analysis must be prepared before start the HPLC analysis. A high-pressure pump is used to delivered the marker compound to detector and generate specified flow rate of mobile phase depends on the marker compound method. An autosampler injector is able to inject the sample continuously sample into the HPLC column. The column contains the chromatographic packing material for separation of compound present in the sample. Then, the next part was detector to detect the separated compound which elute from the HPLC column. Then, mobile phase exits the detector and sent to waste reservoir (Waters, 2017). Many research have been used HPLC quantitative analysis of gallic acid from various sources such as green, Oolong, black and pu-erh teas (Zuo et al., 2002), green tea (Wang et al., 2000), longan seed and mango kernel (Soong & Barlow, 2006) and hazel bark, twig and leaf (Wang et al., 2003).

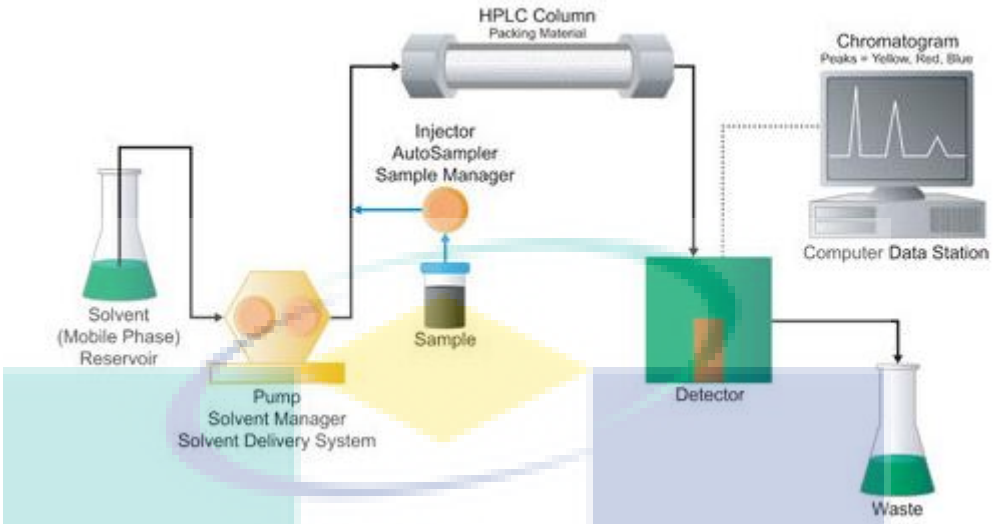


Figure 2.6 High-performance liquid chromatography (HPLC) systems

Source: Waters (2017)

2.4.2 Liquid Chromatography Mass Spectrophotometer-Quadrupole Time-of-Flight (LCMS-QTOF)

There are two common technological platforms used for structure identification which are mass spectrophotometer (MS) and nuclear magnetic resonance (NMR) spectroscopy. However, MS provides more sensitive and larger dynamic range. In LCMS-QTOF analysis, metabolites are characterized refer to the Plant Metabolic Network (PMN) and Metabolite database (METLIN). The fragmentation spectra and retention time can confirm the identity of metabolites (Zhu et al., 2013). Figure 2.7 shows the liquid chromatography mass spectrophotometer-quadrupole time-of-flight (LCMS-QTOF) by Agilent Technologies Model 6520 from Santa Clara, CA, USA (Agilent, 2017).

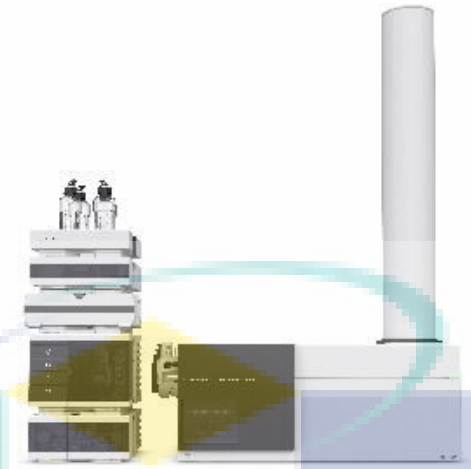


Figure 2.7 Liquid hromatography Mass Spectrophotometer-Quadrupole Time-of Flight (LCMS-QTOF) by Agilent Technologies Model 6520 from Santa Clara, CA, USA

Source: Agilent (2017)

2.4.3 Field emission scanning electron microscopy (FESEM)

Field Emission Scanning Electron Microscope is used to analyze the surface of dried extracted sample the in very smail topogenic detail. A FESEM was works with electrons (particles with a negative charge). These electrons are liberated by a field emission source and accelerated in a high electrical field gradient. This electron called as primary electrons. A detector will produces an electonic signal which transformed to video scan image after catches the secondary electrons. This image will displayed on monitor and digital image can be saved (Geert et al., 2012). Figure 2.8 shows the field emission scanning electron microscope (FESEM) unit by Hitachi Model S-4700 (Acmal, 2014).

Figure 2.8 Field Emission Scanning Electron Microscope (FESEM) unit by Hitachi Model S-4700

Source: Acmal (2014)

2.5 Summary

Ultrasound-assisted extraction (UAE) method improve the extraction process by increasing the rate of extraction, reducing the extraction time and optimizing the yield of extraction (Vilkhu et al., 2008). The UAE method improve the extraction yield by destruct of cell wall barrier, enlarge pores of the cell wall, enhance diffusion, formation and collapse of cavitation bubbles, release of tremendous energy, and enhance diffusion and mass transfer . Whereas, enzymatic extraction (EnE) facilitate cell matrix destruction, hydrolyse the lignin content in cell wall structure and improve the intracellular compound release process from the matrix (Gavit et al., 2011). When EnE and UAE is combined UAE can facilitate both the enzyme reaction and the extraction process (Vinatoru, 2001) as it can improve the cellulase activity rate with low-intensity ultrasound (Wang et al., 2012). Hence, this study introduced the more effective method to extract active compounds from the herbs with the application of low intensity ultrasound on the enzymatic extraction of gallic acid from *Labisia pumila.*

CHAPTER 3

MATERIALS AND METHODS

The materials and methods of the research carried out in this study are discussed in this chapter. The study will focus on the improvement in extraction process of *Labisia pumila* by using:

i. Ultrasound-assisted extraction

ii. Enzymatic extraction

iii. Ultrasound-assisted enzymatic extraction

Three different methods were used in extraction of gallic acid from *Labisia pumila* to identify:

i. Sonication regiments and duty cycle that can promote the process

ii. The suitable concentration of cellulase enzyme to enhance the process

iii. Best combination condition of ultrasound and enzyme that give the highest improvement on the process

3.1 Materials

The plant material, *Labisia pumila* were purchased locally from Delima Jelita, Simpang Empat, Alor Setar, Kedah was used for all experiments. Prior experiment, the *Labisia pumila* was grounded and sieved with particle size range of 0.15 to 0.3 mm by

using a standard sample sieve and stored in the fridge at 4 ^{o}C until it is used for the experiments. Prior to complete the experimental work, chemical involve are sodium acetate and acetic acid for pH adjustment for enzymatic extraction method (Table 3.1).

Table 3.1 Chemical used for enzymatic extraction

Chemical	CAS Number
Sodium acetate	127-09-3
Acetic acid	64-19-7
Cellulase *Tricoderma reesei*	9012-54-8

3.2 Aqueous extraction (AE) of *Labisia pumila*

Aqueous extraction procedure used in this study is summarized in Figure 3.2. Ground *Labisia pumila* leaves were immersed in the extraction solvent and the mixture was heated on a hotplate with continuous stirring for 8 hours with the volume of infusion set at 300 mL (Figure 3.1). The mixture was covered with aluminium foil throughout the extraction to minimize the evaporation in order to maintain the sample-to-water ratio.

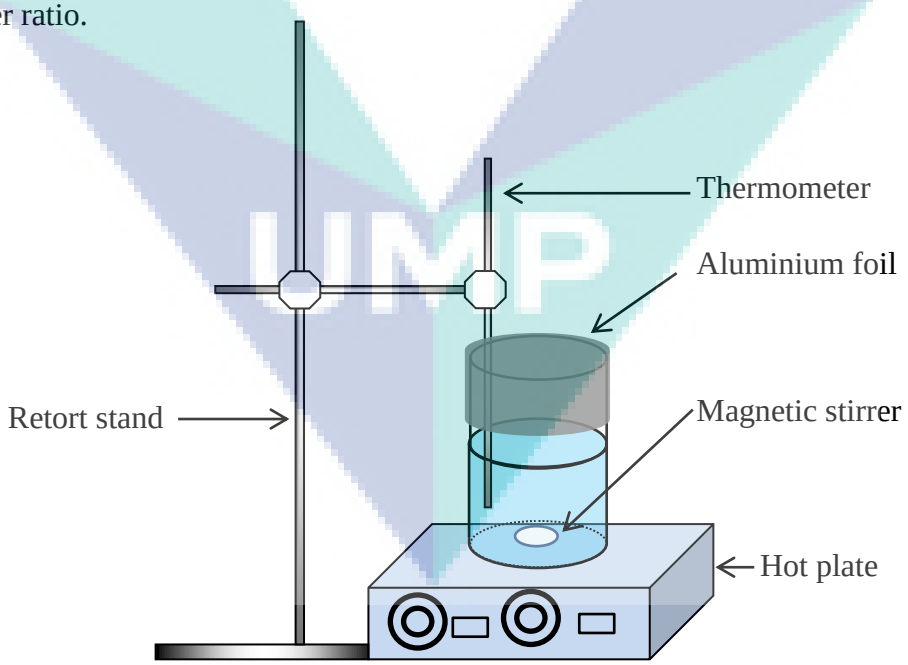


Figure 3.1 Schematic diagram of the aqueous water extraction setup

In aqueous extraction setup, four different extraction temperature were applied (40, 50, 60, and 80 °C) and the sample-to-solvent ratio (wt/wt) (*Labisia pumila* to water) are 1 : 6, 1 :8, 1: 10 and 1 : 12. Then, the sample was centrifuged using centrifuge by Kubota Corporation at 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after extraction process (Table 3.2). Extracted product was left to cool at room temperature and then was kept at 4 ºC prior to analysis. The temperature was measured with an external temperature probe. Each experiment was performed in triplicate. The procedure of the aqueous extraction is summarized in the Figure 3.2.

Table 3.2 Parameter involved in aqueous extraction experiment

Variable	**Details**
Varieties	*Labisia pumila* var. alata
Infusion Volume	300 mL
Particle size	0.15-0.3 mm
Centrifugation speed	5000 rpm for 10 min
Temperature	40, 50, 60, and 80 °C
Sample-to-solvent Ratio	1 : 6, 1 :8, 1: 10 and 1 : 12 (wt/wt)

Cleaned and dried *Labisia pumila* is grounded and sieved with particle size range of 0.15 to 0.3 mm by using a standard sample sieve and a sieve shaker.

Grounded *Labisia pumila* leaves were immersed in the extraction solvent and the mixture was heated on a hotplate with continuous stirring

1) Time : 8 h

2) Temperature : 40,50,60, and 80 °C

3) Sample-to-solvent ratio (sample : water) : 1 : 6, 1 :8, 1: 10 and 1 : 12(wt/wt) with the volume of infusion was set at 300 mL.

Centrifuged with speeds 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after extraction process. Extracted product was left to cool at room temperature and then was kept at 4 °C prior to analysis

Analysis:

1) Determination of gallic acid yield by using HPLC-DAD at wavelength 270 nm;

2) Identification of gallic acid metabolite by using LCMS-Q-TOF analysis;

3) Morphological study on the extracted *Labisia pumila* by FE-SEM observation.

Figure 3.2 Aqueous extraction procedures

3.3 Ultrasound-assisted extraction (UAE) of *Labisia pumila*

Ultrasound-assisted extraction of gallic acid from *Labisia pumila* (Figure 3.5) was conducted by using ultrasonic processor Q700 (700 W, 20 kHz) from QSonica, Newtown, U.S.A with a replaceable flat tip ultrasonic probe (sonotrode) made of titanium alloy that had a tip diameter of 12.7 mm and 50 mm length (Figure 3.3).

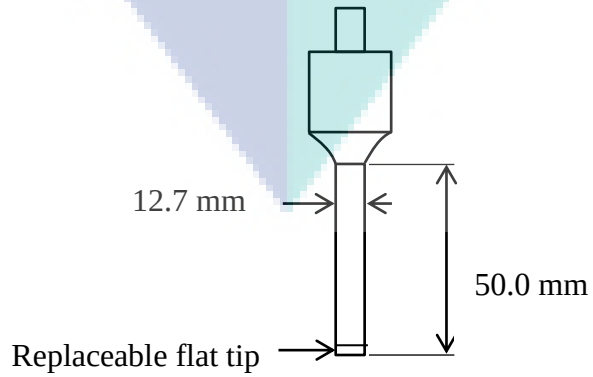


Figure 3.3 Dimension of ultrasonic probe (sonotrode)

The ultrasonic probe was immersed in the extraction medium and the energy is transmitted via the sonotrode directly into the sample. The ultrasound power level was fixed by setting the amplitude of the sonotrode and the cumulative average ultrasound dose by adjusting the duty cycle. The sonication intensity was calculated using the following equation:

$$I = \frac{P}{A} \qquad 3.1$$

where A is the area of the sonotrode tip and P is the ultrasound power. The control (aqueous extraction) experiments did not use sonication although the sonotrode was installed as in Figure 3.4.

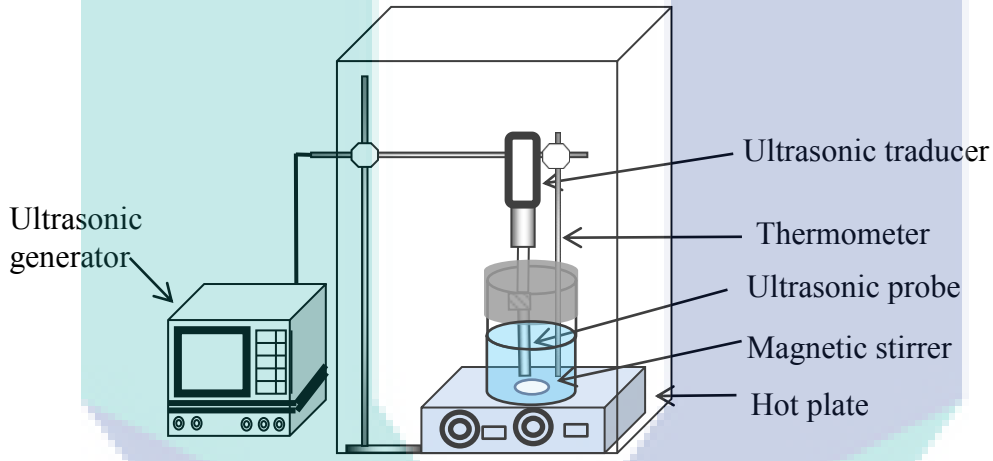


Figure 3.4 Schematic diagram of ultrasound-assisted extraction

For UAE (Table 3.3), four different extraction temperature were applied (40, 50, 60, and 80 °C) and the sample-to-solvent ratio (*Labisia pumila* : water) of 1: 10 is used. The amplitude was set at position 1 to correspond to a power input P of 11 W, and of 8.66 W/cm^2 sonication intensity, I using 40 % duty cycles (A duty cycle of 40%, for example, was obtained by sonication for 4 s followed by a rest period of 6 s) (Table 3.4). Then, the sample is centrifuged at 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after extraction process. Extracted product was left to cool at room temperature and then was kept at 4 °C prior to analysis. The temperature was measured with an external temperature probe. Each experiment was performed in triplicate.

Table 3.3 Parameter involved in UAE experiment

Variable	Details
Varieties	*Labisia pumila* var. alata
Infusion Volume	300 mL
Particle size	0.15-0.3 mm
Centrifugation speed	5000 rpm for 10 minutes
Temperature	40, 50, 60, and 80 °C
Sample-to-solvent Ratio	1 : 10 (wt/wt)
Ultrasound intensity	8.66 W/cm^2
Amplitude	1
Duty cycle	10, 20 and 40 %

Table 3.4 Sonication regimens used at a fixed sound intensity 8.66 W/ cm^2

Duty cycle	Pulse ratio
10	Sonication for 1 s followed by rest period (no sonication) of 9 s
20	Sonication for 2 s, rest of 8 s
40	Sonication for 4 s, rest of 6 s

*Duty cycle has been set up using generator from the system

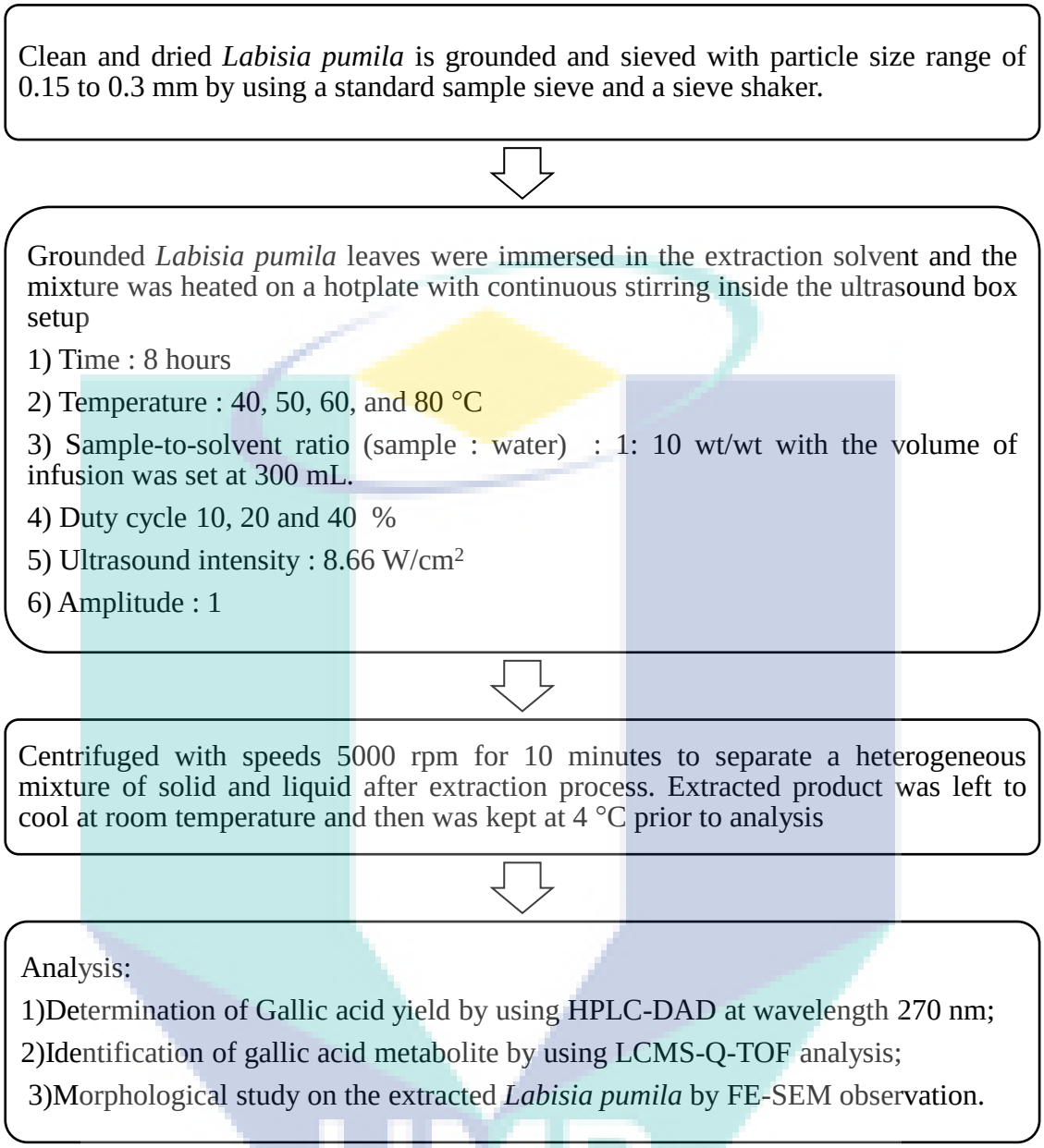


Figure 3.4 Ultrasound-assisted extraction procedures

3.4 Enzymatic extraction (EnE) of *Labisia pumila*

Enzymatic extraction of *Labisia pumila* is summarized in Figure 3.7. The enzymatic hydrolysis is conducted using cellulase as biocatalysis throughout of experiments (Figure 3.6). The operating conditions, including extraction time, temperature, and sample-to solvent ratio is identical with UAE method (Table 3.5). The difference is only the sample immersed in cellulase solution with varies concentration and incubation in a water bath. Commercial enzymes,; cellulase *Tricoderma reesei*

(Table 3.5), with unit activity ≥700 units/g are used during the study and the enzymes were kept at 4-8 ^{o}C. Figure 3.7 shows the procedure for the process.

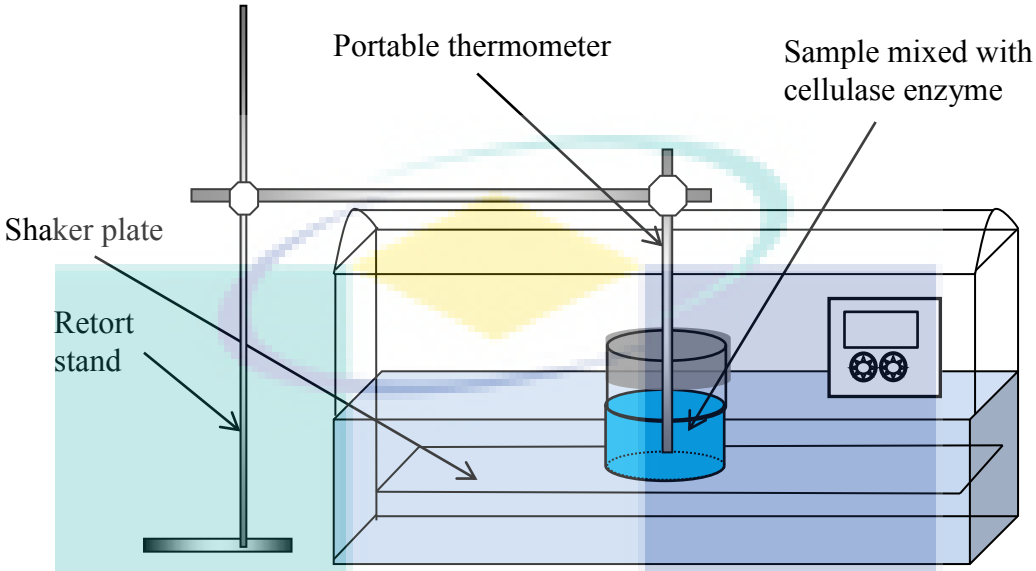


Figure 3.5 Schematic diagram of enzymatic extraction

In enzymatic extraction procedure,five different enzyme concentration were applied (0.025, 0.050, 0.1, 0.2, and 0.3 g/L) and the sample-to-solvent (wt/wt) ratio *(Labisia pumila* : water) of 1: 10 is used. The 0.05 M acetate buffer (Shoemaker et al., 1983) wash prepared and acetic acid was added to the mixture to adjust the pH condition. Optimum temperature of cellulase (50 °C) is used with continuous shaking for 8 hours in the water bath as shown in Figure 3.6. Then the mixture was boiled at 100 ºC for 5 minutes to stop the enzyme activity. Then, the sample is centrifuged at 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after extraction process. Extracted product was left to cool at room temperature and then was kept at 4 ºC prior to analysis. The temperature was measured with an external temperature probe. Each experiment was performed in triplicate.

Table 3.5 Parameter involved in enzymatic extraction experiment

Variable	Details
Varieties	*Labisia pumila* var. alata
Infusion Volume	300 mL
Particle size	0.15-0.3 mm
Centrifugation speed	5000 rpm for 10 minutes

Table 3.6 Continued

Variable	Details
Temperature	50 °C for 8 hours
40, 50, 60, and 80 °C for 4 hours	
Sample-to-solvent Ratio	1 : 10 (wt/wt)

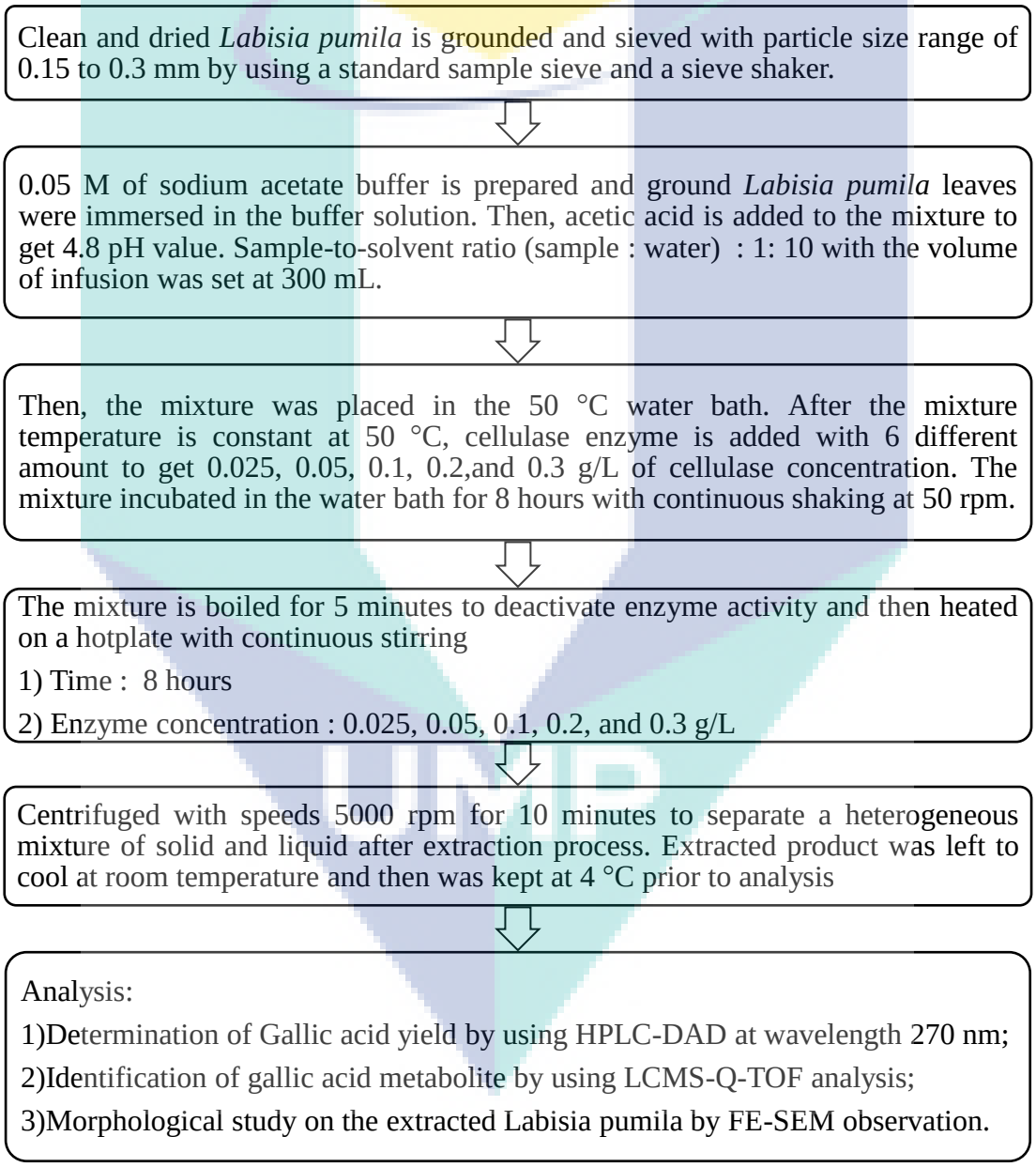


Figure 3.6 Enzymatic extraction procedures

3.5 Ultrasound-assisted enzymatic extraction of *Labisia pumila*

The ultrasound enzymatic assisted extraction is conducted by using ultrasound probe from Misonix Sonicators Model Q700 and commercial enzymes cellulase Tricoderma reesei with unit activity ≥700 units/g from Sigma-Aldrich**.** For this study, best sonication regimens selected from UAE and best enzyme concentration from enzymatic extraction is unified for the extraction of gallic acid from *Labisia pumila*. Figure 3.8 shows the experimental setup for the process.

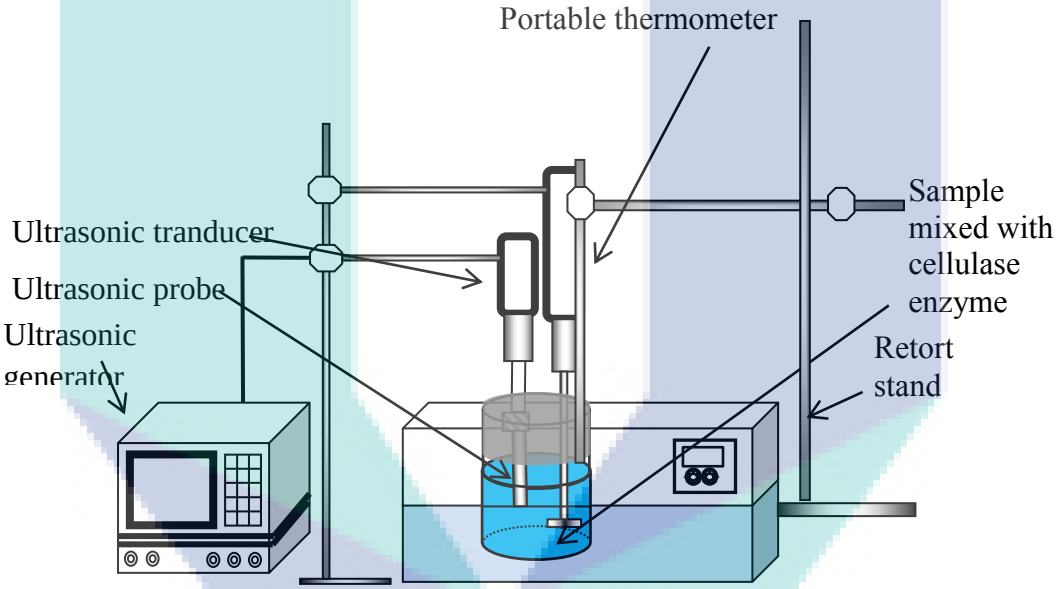


Figure 3.7 The experimental setup for ultrasound-assisted enzymatic extraction

In ultrasound-assisted enzymatic extraction procedure (Figure 3.9), optimum temperature of cellulase (50°C) is used with continuous shaking for 8 hours in the water bath (Table 3.6). Then, the sample is centrifuged at 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after extraction process. Extracted product was left to cool at room temperature and then was kept at 4 °C prior to analysis. The temperature was measured with an external temperature probe. Each experiment was performed in triplicate.

Table 3.7 Parameter involved in ultrasound-assisted enzymatic extraction experiment

Variable	Details
Varieties	*Labisia pumila* var. alata
Infusion Volume	300 mL
Particle size	0.15-0.3 mm
Centrifugation speed	5000 rpm for 10 minutes
Temperature	50 °C for 8 hours
Sample-to-solvent Ratio	1 : 10 (wt/wt)
Duty cycle (%)	40 (Sonication for 4s followed by rest period (no sonication) of 6 s
Cellulase concentration	0.05 g/L

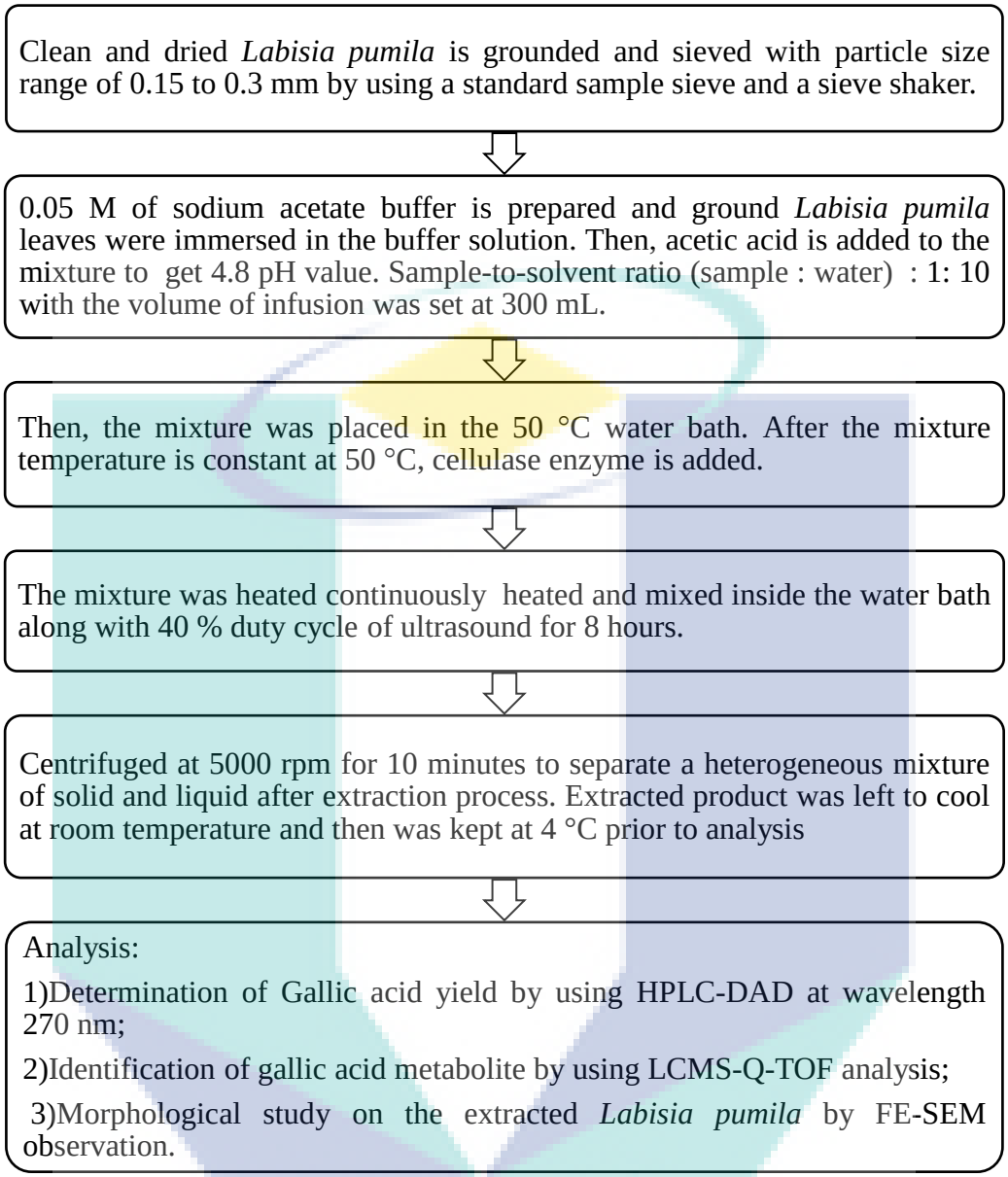


Figure 3.8 Ultrasound-assisted enzymatic extraction procedures

3.6 Determination of Gallic acid yield by high performance liquid chromatography (HPLC-DAD) Analysis

To measure the amount of gallic acid yield from the extract, the sample was analysed using Method of high performance liquid chromatography (HPLC) analysis from Malaysian Standard. The high performance liquid chromatographic used was by Agilent Technology 1100 Series (Model G1379A) analysis using method for analyse gallic acid as tabulated in Table 3.7. The detail of the procedure is described in Figure 3.11. Reverse-phase C-18 column was used to separate and quantitate the gallic acid

with an isocratic elution system of acetonitrile/phosphoric acid by UV detector (Table 3.8). The chemicals used for HPLC analysis is illustrated in Table 3.8

Table 3.8 Method of high performance liquid chromatography (HPLC) analysis

Column	C18 column, 4.6mm x 250mm ,5µm particle size or equivalent
Mobile phase	A : Acetonitrile B : 3% Phosphoric buffer water
Analysis mode	Isocratic (Solvent system A/B=10/90)
Flowrate	1mL/min
Wavelength	UV at 270 nm
Retention time	4-7 minutes
Run time	10 minutes

*This method was the standard method for gallic acid from Malaysian Standard, 2013

Table 3.9 Chemical used for HPLC-DAD analysis

Chemical	CAS Number
Gallic acid (analytical grade)	149-91-7
Phosphoric Acid (analytical grade)	7664-38-2
Acetonitrile (gradient grade for liquid chromatography)	75-05-8

The standard curve of gallic acid concentration is prepared (Figure 3.10) as a reference to calculate the amount of gallic acid present in the sample. Then, the gallic acid concentration of each sample can be calculated by using the calibration curve equation. However, this is not the final concentration of the gallic acid, the actual amount of gallic acid can be calculated using the equation 3.1. Then, the sample preparation procedures for HPLC-DAD analysis is summarized in Figure 3.11.

$$\%\frac{w}{w} = \frac{CxVx\ DF}{W}\ x\ 100 \qquad 3.2$$

Where

C	Calculated concentration from HPLC chromatogram, ppm
V	Volume of solvent, L
DF	Dilution factor
W	Weight of sample, kg

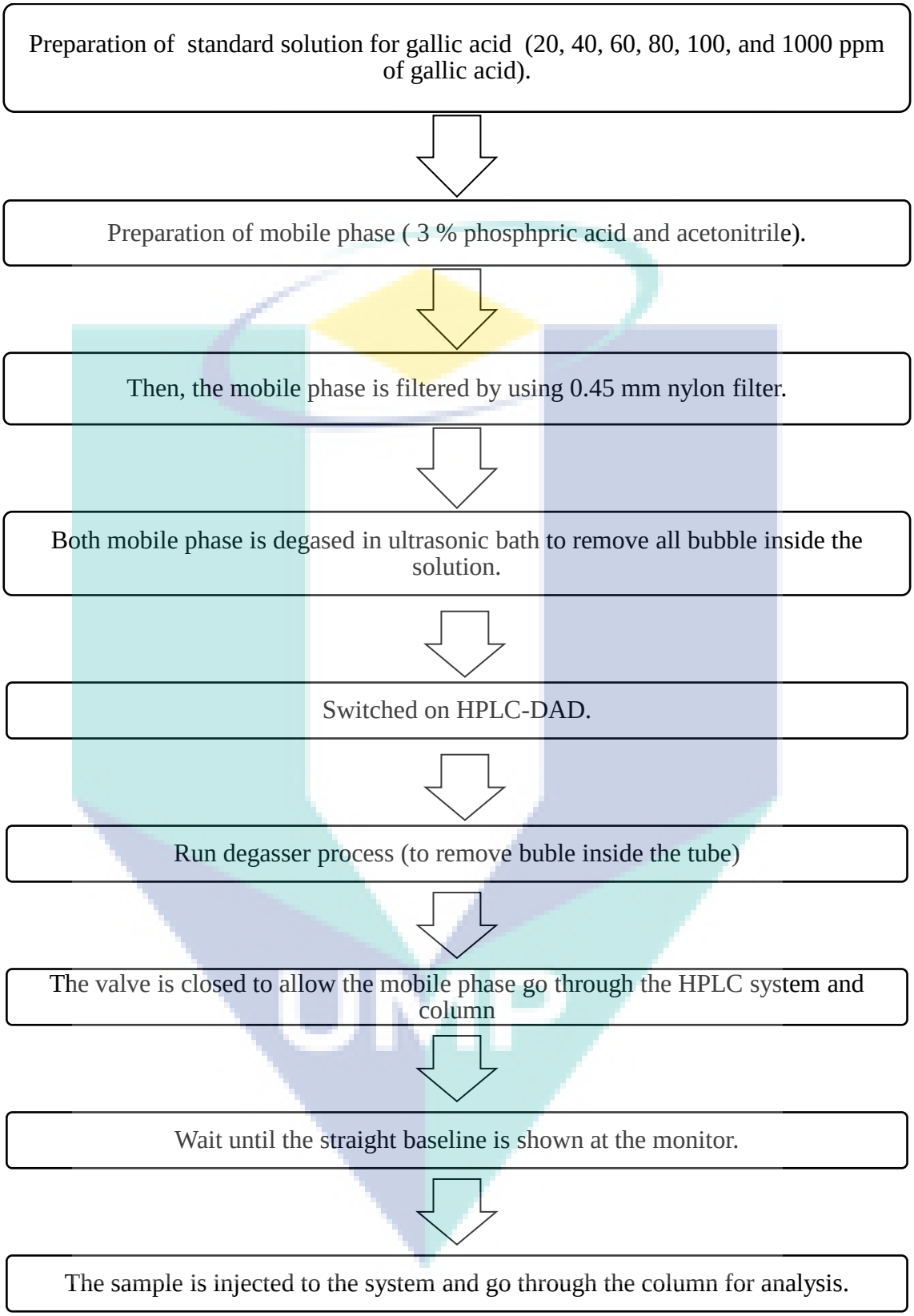


Figure 3.9 HPLC-DAD analysis procedures for preparation of standard gallic acid curve

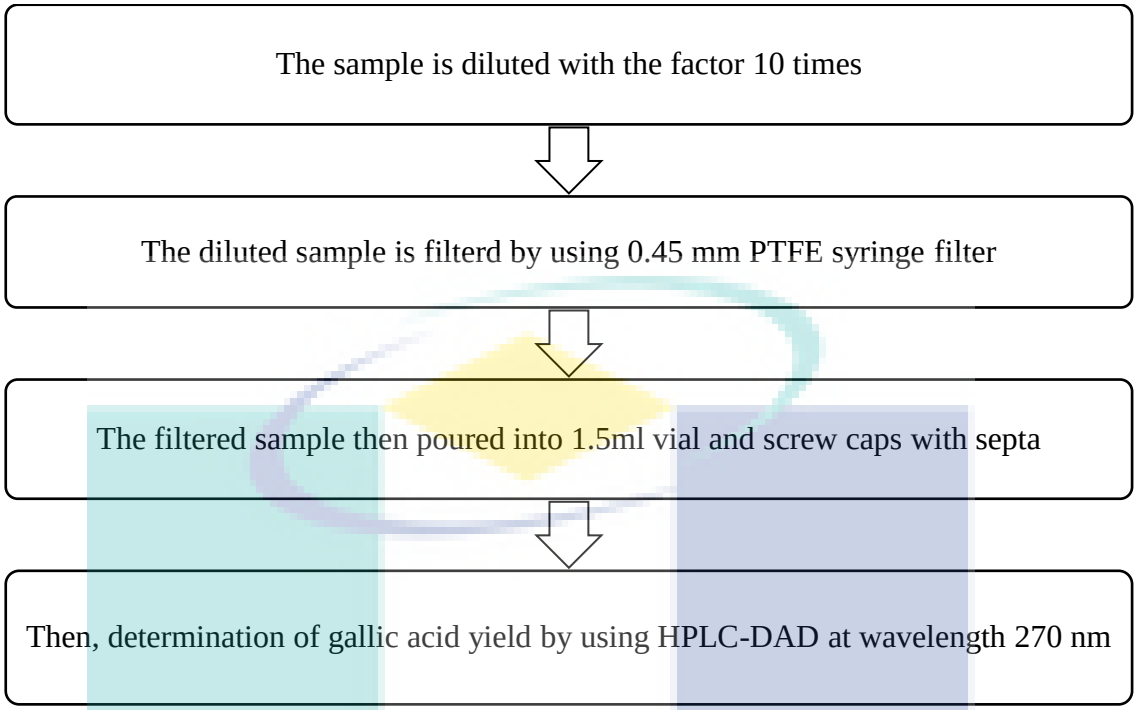


Figure 3.10 Sample preparation procedures for HPLC-DAD analysis

3.7 Glucose concentration determination by using UV-Vis Spectrophotometer

To measure the amount of glucose concentration present in the enzymatic extraction, the sample was analysed using UV-Vis spectrophotometer by Hitachi model U-1800).The Dinitrosalicylic acid (DNS) reagent is prepared according to Saqib and Whitney, 2011and the procedure detail of is described in Figure 3.12. Then, the sample is analysed at 575nm absorbance as illustrated in Figure 3.13. The chemical used in UV-Vis spectrophotometer analysis is tabulated in Table 3.9

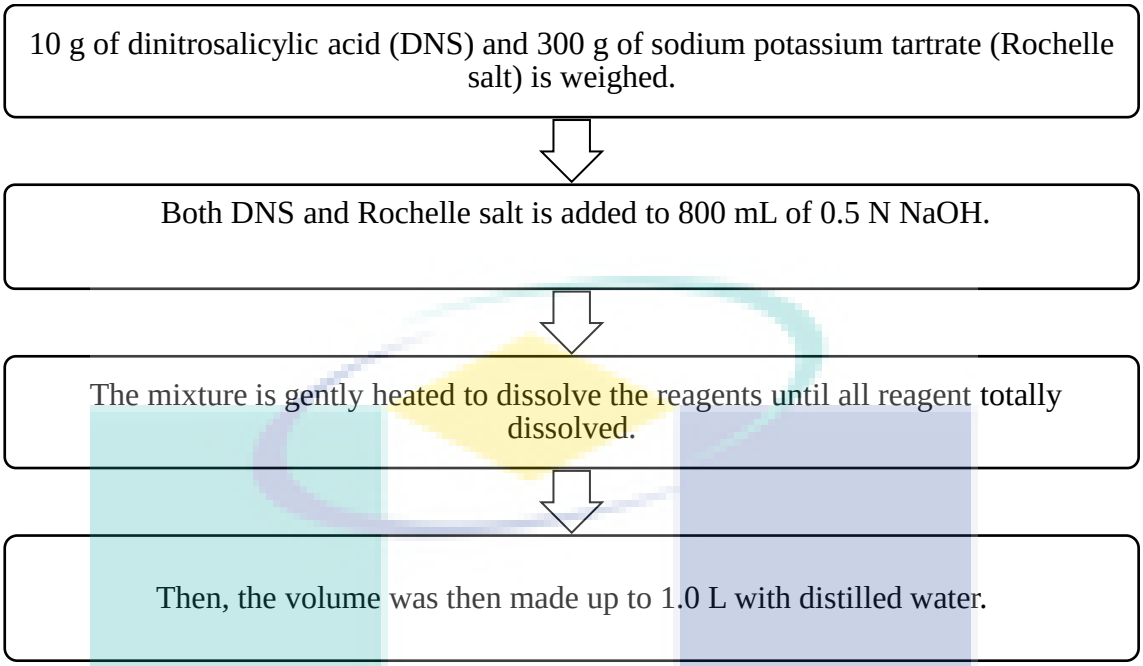


Figure 3.11 The procedure to prepare dinitrosalicylic acid (DNS) reagent

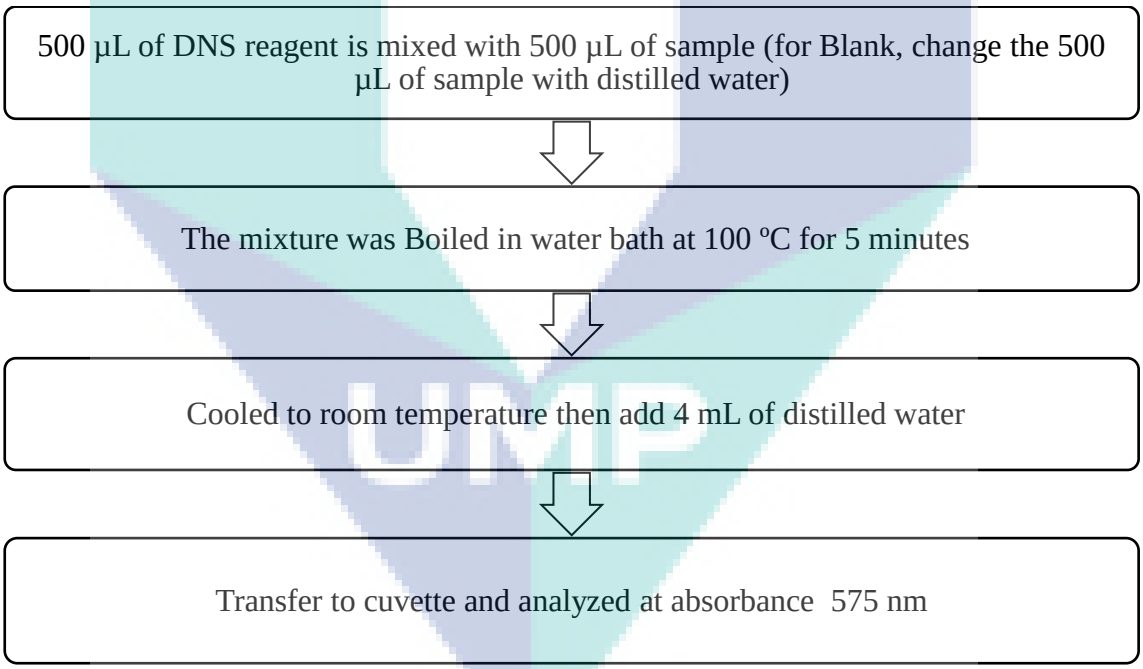


Figure 3.12 The UV-Vis spectrophotometer procedure analysis

Table 3.10 Chemical used for UV-Vis Spectrophotometer

Chemical	CAS Number
Dinitrosalicylic acid (DNS) (Analytical grade)	609 99 4
Potassium tartrate (Rochelle salt) (Analytical grade)	6381 59 5

3.8 Determination of gallic acid liquid chromatography mass spectrophotometer-quadrupole time-of-flight (LCMS-QTOF)

The LCMS/MS Q-TOF (Agilent Technologies 6520, Santa Clara, CA, USA)was used to obtain the MS and MS/MS data. The mobile phase used is highly purified acetonitrile. Column used was 2.1× 100 mm ZORBAX Eclipse plus 18 column. The flow rate was set up at 0.6 mL/min with 47 minutes total run time. The prepared samples were placed into the LCMS autosampler. Analysis was performed in negative ion mode. The mass range was at 110–350 m/z. This analysis was performed by Integrative Pharmacogenomics Institute (IPROMISE),Universiti Teknologi MARA (Puncak Alam , Selangor Malaysia).

3.9 Morphological study by field emission scanning electron microscope (FESEM)

For FESEM analysis, the extracted sample is dried in the oven at 60 ºC for 8 hours to ensure the sample is totally dried and suitable for FESEM analysis. This analysis was performed by Central Laboratory, Universiti Malaysia Pahang (Gambang, Pahang Malaysia).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

In this chapter, data from experimental studies were presented and discussed. Four different procedures were used for this experiment. This chapter begins with the discussion on the effect of temperature and sample-to-solvent ratio on the conventional aqueous extraction (AE) of gallic acid from *Labisia pumila*. Then, continued with the effect of sonication regimens toward the ultrasound-assisted extraction (UAE) process. The next part of this chapter will show the influence of enzyme concentration on the enzymatic extraction (EnE) of gallic acid from *Labisia pumila*. Followed by, the enhancement of unified ultrasound-assisted enzymatic extraction (UAEnE) procedure on the gallic acid yields throughout the process. The results were analysed by HPLC-DAD quantitatively, LCMS-Q-TOF for qualitative analysis and FESEM analysis to study the physical effect on the extracted *Labisia pumila* sample.

4.2 Gallic Acid Standard Curve

This method is ideally suited for gallic acid determination with good repeatability and accuracy of results. Figure 4.1 shows the calibration curve of gallic acid in the range of 5-80 mg/L concentrations with excellent regression. Hence, the equation transform from the line will be used to calculate the gallic acid concentration in the sample.

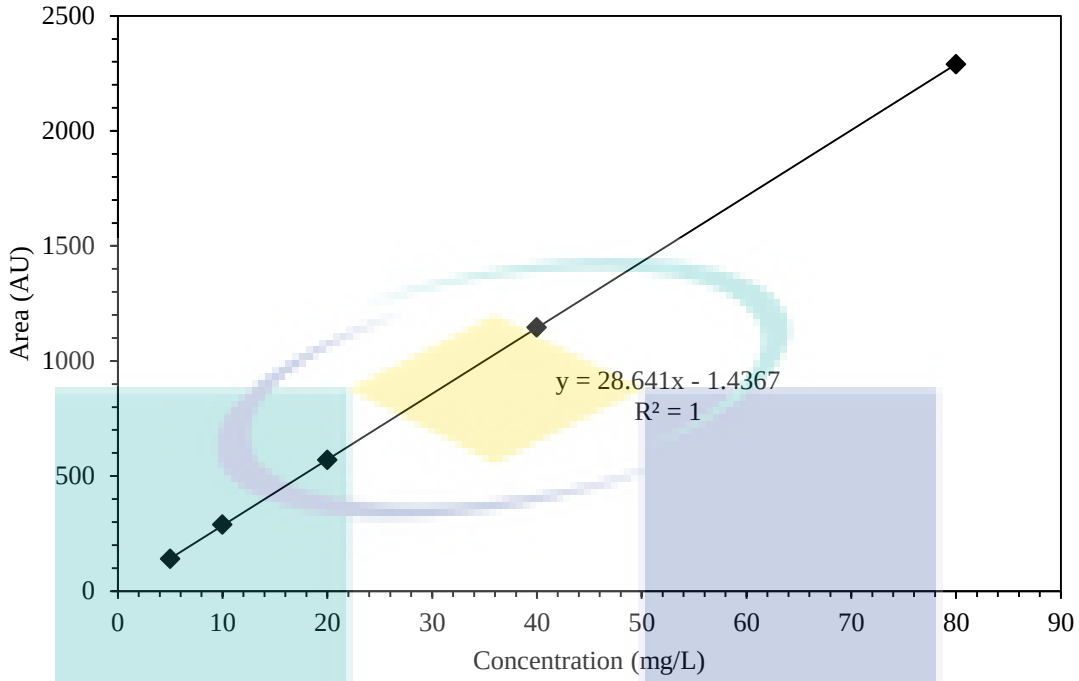


Figure 4.1 Calibration curve of gallic acid

4.3 Baseline determination (aqueous extraction)

The result of aqueous extraction is used as baseline data for comparison with the UAE, EnE and UAEnE. In this study, sample-to-water ratios of 1:6, 1:8, 1:10, and 1:12 (g/mL) were initially utilized at 60 °C. The extraction was essentially done by 8 hours and Figure 4.2 shows the comparison effect of sample-to-water ratio on the average gallic acid yield from extract (8 hours, 60 °C) and Figure 4.3 shows the gallic acid yield values extracted after 4 h and 8 hours extraction at 1:6, 1:8, 1:10 and 1:12 sample-to-solvent ratio. At sample-to-water ratio of 1:10 was recorded to have the highest gallic acid yield, 0.9530 ± 0.0377 mg/g, after 8 hours of extraction . The amount of gallic acid yield from *Labisia pumila* was increased when the ratio increased from 1:6 to 1:10 (0.8677 ± 0.0399 < 0.8825 ± 0.0296 < 0.9530 ± 0.0377 mg/g). However, the yield was slightly decreased to 0.9311 ± 0.0082 mg/g when the sample-to-water ratio was increased to 1:12. This indicated that ratio 1: 10 is the most ideal ratio to release the active compound from *Labisia pumila*. These results have followed the similar trend obtained by Palma et al. (2013). The amount of water used for the extraction process was enough to release the desired active compound. Hyun-kyung Choi et al. (2010) and Zulkarnaini et al. (2013), used the sample-to-water ratio of 1: 10 in their study on the

extraction of active compound from *Labisia pumila.* Hence, sample-to-water ratio of 1:10 is selected throughout this study.

In this study, sample of *Labisia pumila* were used in powder forms. The particle size was determined in the range of 0.15 to 0.3 mm by sieving using a standard sample sieve and a sieve shaker. Ground *Labisia pumila* leaves were immersed in the extraction solvent and the mixture was heated on a hotplate with continuous stirring for 8 hours. Four different extraction temperature were applied in this study (40, 50, 60, and 80 °C) and the sample-to-solvent ratio of each mixture was set at 1: 10 (sample: water) with the volume of infusion was set at 300 mL. The mixture was covered with aluminium foil throughout the extraction to minimize the evaporation in order to maintain the sample-to-water ratio. Then, the sample is centrifuged at 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after the extraction process. Extracted product was left to cool at room temperature and then was kept at 4 °C prior to analysis for determination of active compounds by using HPLC. The temperature was measured with an external temperature probe. Each experiment was performed in triplicate.

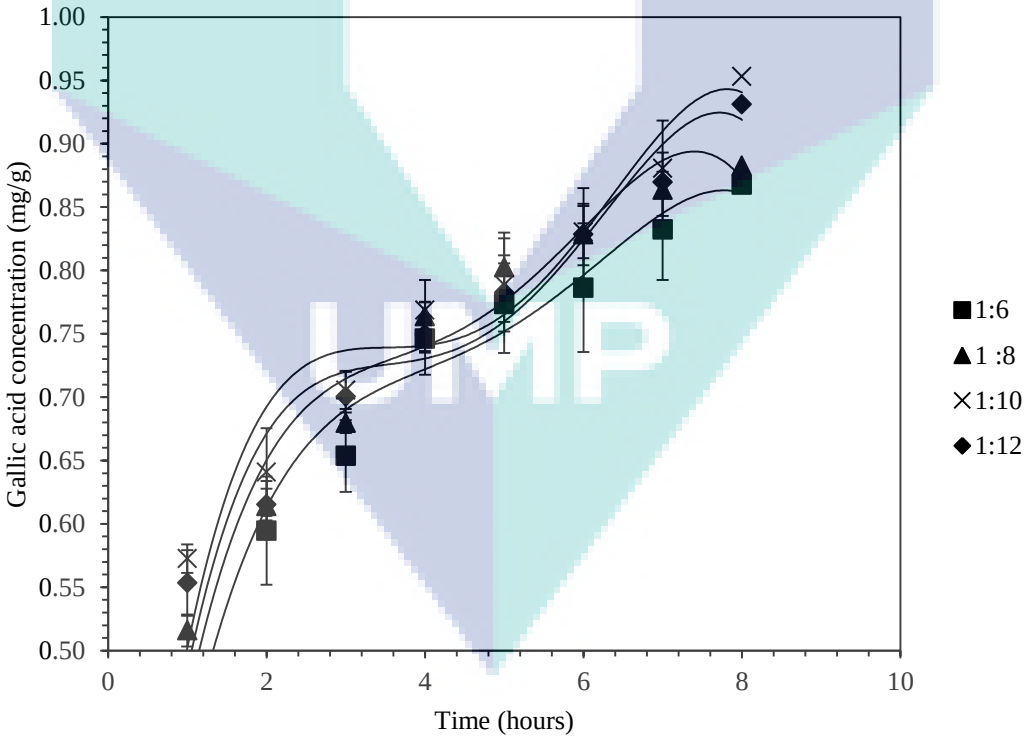


Figure 4.2 Comparison effect of sample-to-water ratio on the average yield of gallic acid from extract (8 hours, 60 °C)

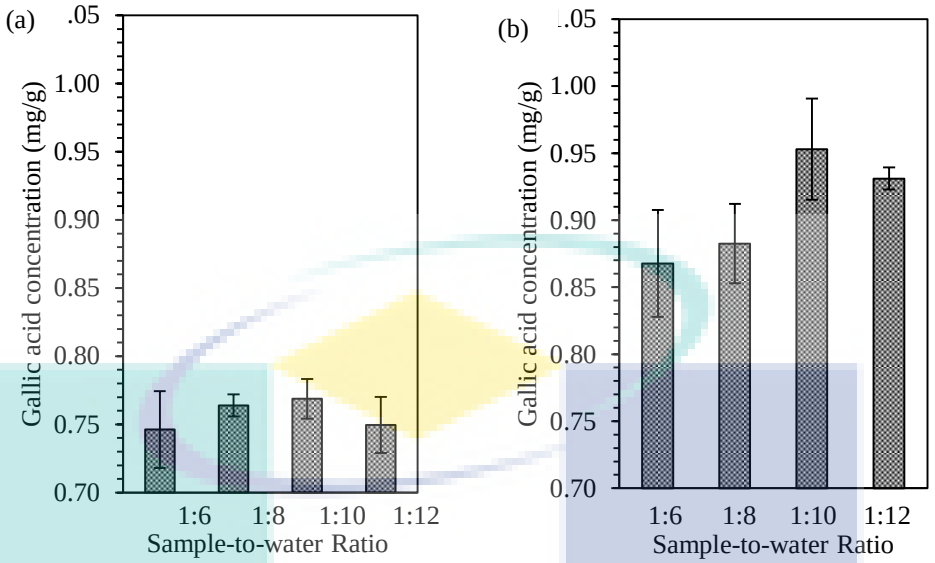


Figure 4.3 Effect of sample-to-water ratio on the average gallic acid yield from *Labisia pumila* extract at 60 °C for (a) 4hours extraction and (b) 8 hours extraction at 1:6, 1:8, 1:10 and 1:12 sample-to-solvent ratio

In determining the effect of extraction temperature, 4 different temperatures (40, 50, 60 and 80 °C) were monitored for 8 hours. Figure 4.4 and 4.5 were plotted to indicate the comparison effect of temperature 40, 50, 60, and 80 °C on the gallic acid yield. The sample-to-water ratio was fixed at 1:10. The increasing pattern was observed for all temperature from 0 to 8 hours of extraction. The highest yield was recorded at 50 °C with gallic acid yield of 1.0251±0.0569 mg/g. As temperature increased from 40 to 50 °C, the gallic acid yield was increased by 1.14 fold (0.8958 ± 0.0996 to 1.0251±0.0569 mg/g). In contrary, when the temperature was increased to 60 and 80 °C, the value of gallic acid yield was decreased to 0.9218±.0237 and 0.7990±0.0145 mg/g, respectively. Overall observation for this result can be concluded that the gallic acid yield increasing from temperature 40 to 50 °C, then decreasing trend from temperature 50 to 80 °C. Hence, 50 °C was an ideal temperature for the extraction of gallic acid yield from *Labisia pumila*. An appropriate explanation for this trend is that the more energy was supplied when the higher temperature was applied. This shows that the higher temperature can overcome more bonds present in the cell wall. Besides that, Daneshfar et al. , (2008) pointed that elevated temperature enhance the gallic acid solubility in water. However, when the temperature increased to 60 and 80 °C, the

amount of gallic acid yield extracted was decreased. Palma et al., (2013) claimed that the moderate temperature (less aggressive condition) is more suitable for extraction of natural product to avoid degradation of intracellular constituents. Degradation of gallic acid compound occurred when the temperature applied more than 50 °C.

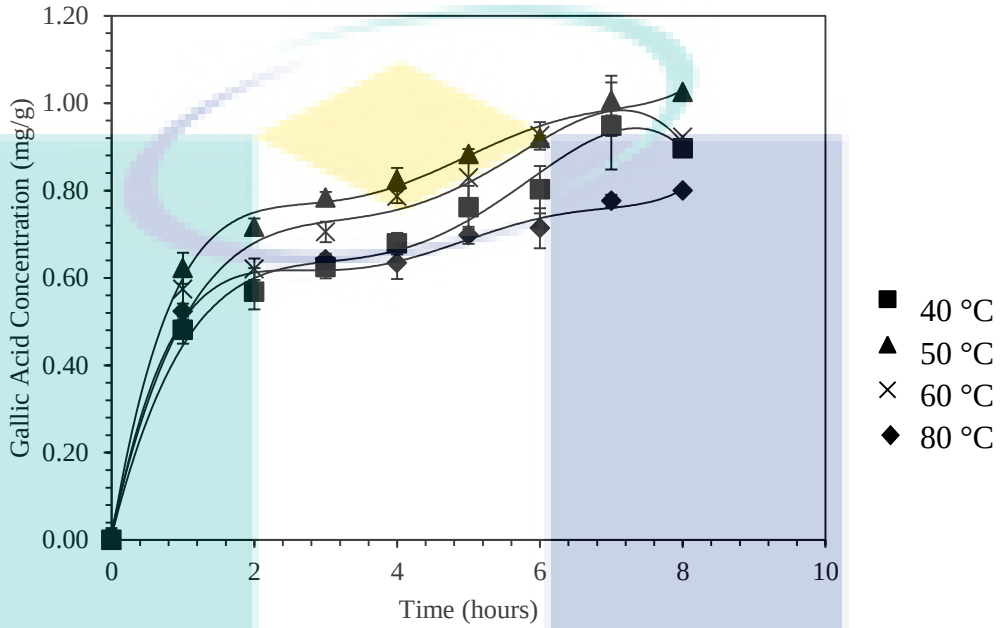


Figure 4.4 Comparison effect of temperature 40, 50, 60, and 80 °C on the average gallic acid yield from extract (1: 10 sample-to-water ratio, 8 hours)

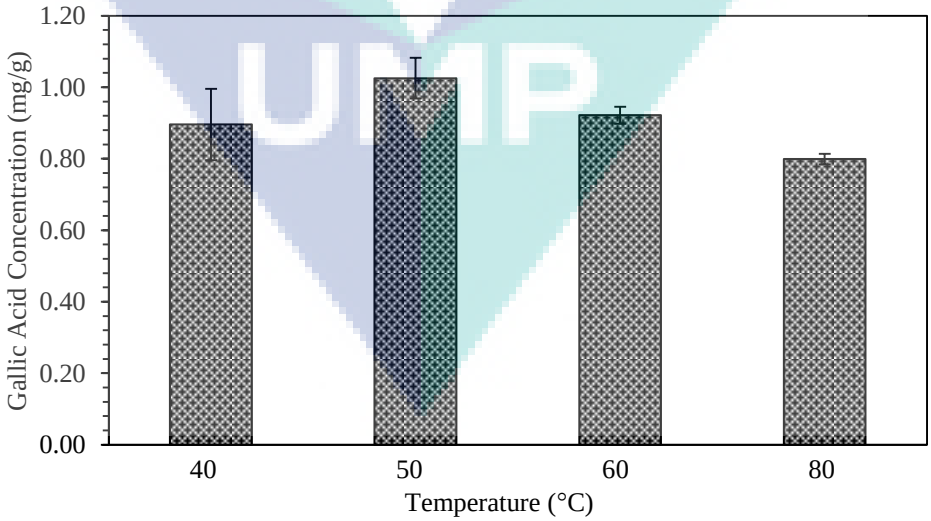


Figure 4.5 Effect of temperature 40, 50, 60, and 80 °C on the average gallic acid yield from extract after 8 hours extraction at 1: 10 sample-to-water ratio

Based on the previous results, 1:10 sample-to-water ratio was selected to study the effect of extraction time at temperature 40, 50, 60 and 80 °C. Figure 4.6 shows the effect of extraction time on the amount of yield released. Overall description for Figure 4.5, the gallic acid yield increased as the time increased. However, the trend in Figure 4.5 shows that the rate of extraction at temperature 50 was the highest with 0251±0.0569 mg/g gallic acid extracted after 8 hours. However, increasing the temperature to 60 and 80 °C showed the decreasing trend of gallic acid yield. As reported by Spigno et al., 2007, mild temperature was more preferred to enhance the extraction process by softening the plant tissues and weaken the cell wall bonding. The active compounds was denatured at high temperature as claimed by Palma et al. (2013), and that the moderate temperature (less aggressive condition) is more suitable for extraction of natural product to avoid degradation of intracellular constituents.

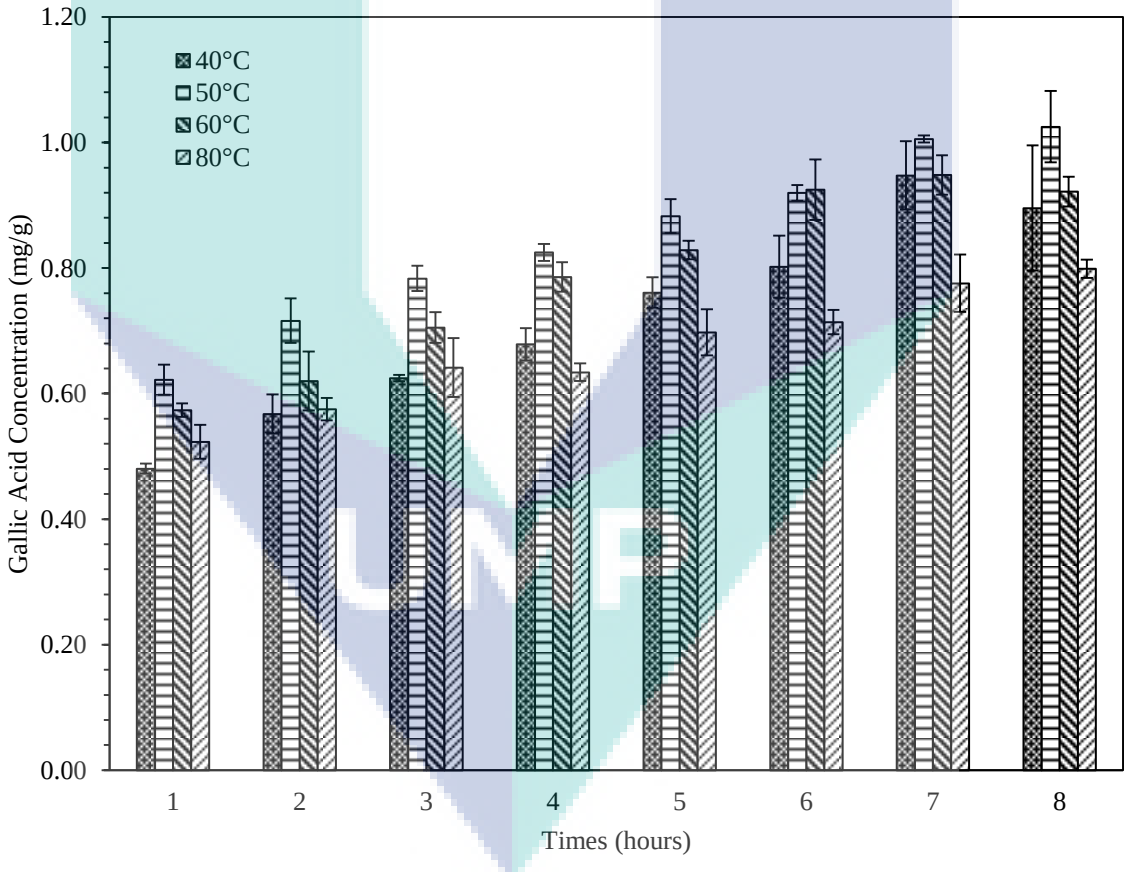


Figure 4.6 Effect of time on the average gallic acid extraction rate at temperature 40, 50, 60, and 80 °C from (1 : 10 sample-to-water ratio)

4.4 Ultrasound-assisted extraction (UAE)

Upon understanding the ultrasound effect on the extraction process, the study was further conducted to determine the effect and the enhancement of sonication regiment towards extraction performance of gallic acid from *Labisia pumila* by comparing the result obtained with control sample / aqueous extraction (AE). Figure 4.7 shows a typical trend of the extraction enhancement by the ultrasound irradiation at difference level of duty cycle except for control (no sonication). The extraction performance is increasing with the increasing of duty cycle from 0% duty cycle (no sonication) to 40 %. This proved that the sonication was improved the extraction of Gallic acid from *Labisia pumila* performance. However, when the duty cycle is higher than 40 %, the gallic acid concentration in the aqueous extracts shows decreasing trend. This shows that, the gallic acid structure was damaged and interrupted by the sonication power. Gallic acid cannot withstand with the sonication power more than 40 % of duty cycle. From the graph, the best duty cycle for the extraction enhancement is 40 % with the highest improvement by 1.13 fold compared to 0 % of sonication. Hence, to study the effect of temperature for ultrasound-assisted extraction, only four duty cycle regimens (0, 10, 20 and 40 %) were employed at constant amplitude 1 s^{-1} and sample-to-water ratio 1:10. The control sample was not subjected to the ultrasound irradiation.

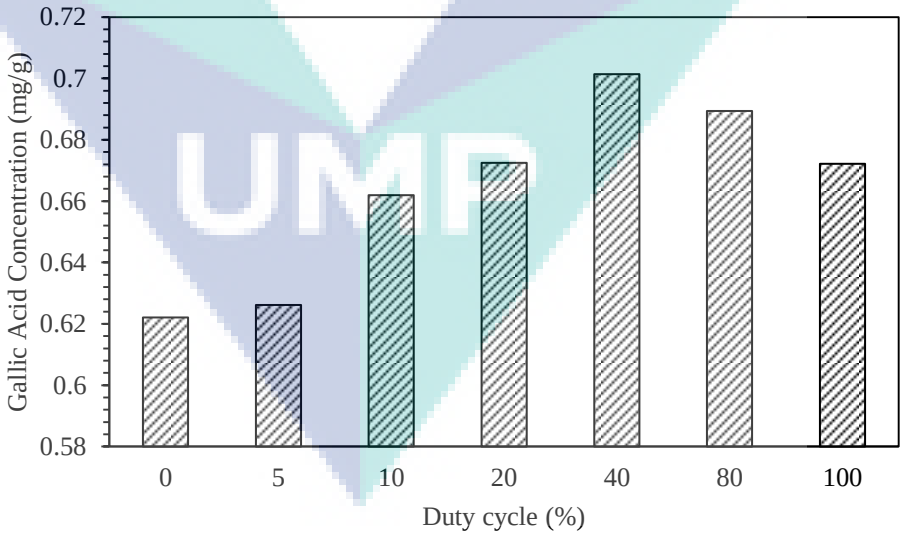


Figure 4.7 Effect of 0 (no sonication), 5, 10, 20, 40, 80 and 100 % sonication duty cycle on the ultrasound-assisted extraction (UAE) at constant temperature.

Ultrasonication is known to improve mass transfer in extraction process. Ashokkumar et al, (2007) and Sulaiman et al, (2011) pointed that sonication enhance the mass transfer at power intensity 2.2 and 11.8 W/cm^2, respectively. For this study, the amplitude is kept constant at 1 to obtain intensity of 8.66 W/cm^2. The lower ultrasound power intensity can promote the extraction process of intracellular constituents from *Labisia pumila*. Hence, the amplitude 1 (8.66 W/cm^2 sonication power) is selected to be applied in the next extraction methods. Figure 4.8, 4.10 and 4.12 shows the effect of different temperature at intermittent mode 10, 20 and 40 % of duty cycle in ultrasound-assisted extraction. The adverse effect of sonication at 10 % duty cycle was plotted in Figure 4.8 and 4.9. Increasing trends was shown as the time increased at all temperature. Prolonged extraction time enhanced the gallic acid yield. The highest gallic acid yield was recorded at 50 °C with the value of 1.6633±0.0688 mg/g, which is equal to 1.62 fold increment compared to control sample.

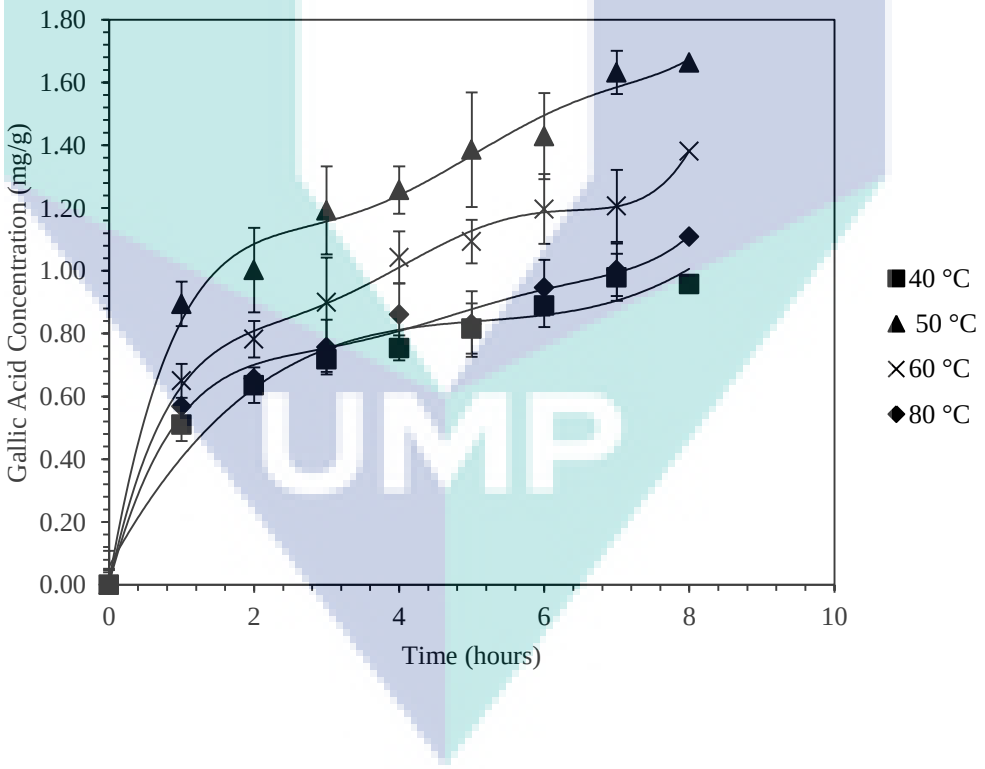


Figure 4.8 Effect of temperature 40, 50, 60, and 80 °C on the ultrasound-assisted extraction (UAE) with 10 % duty cycle in 8 hours

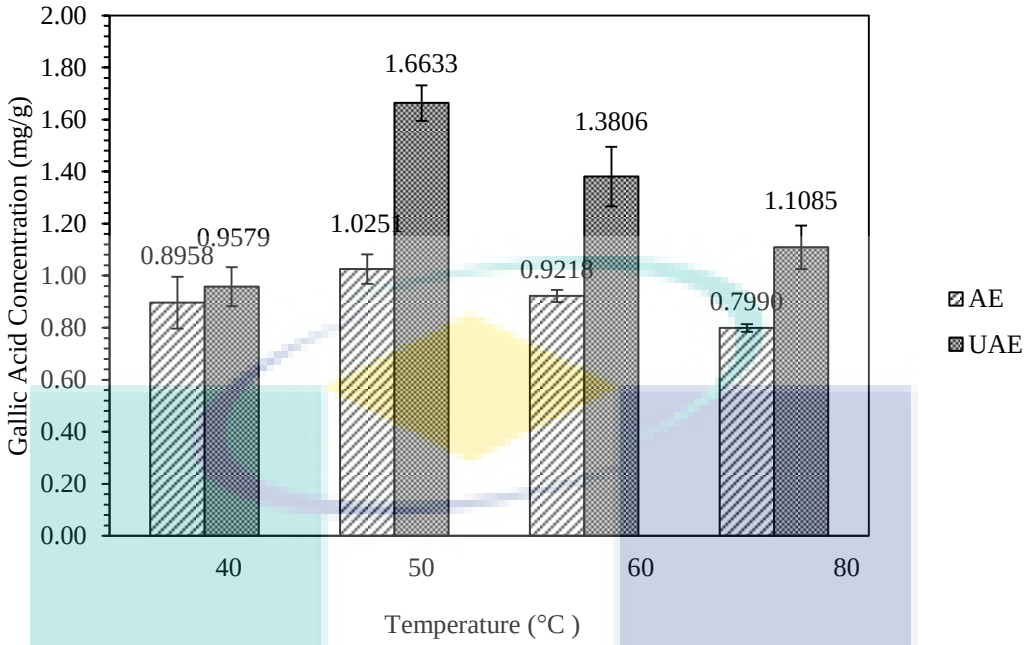


Figure 4.9 Comparison effect of conventional aqueous extraction (AE) and 10 % duty cycle of ultrasound-assisted extraction (UAE) at temperature 40, 50, 60, and 80 °C on the average gallic acid yield by 8 hours extraction and 1 : 10 sample-to-water ratio

The result for 20 % duty cycle of sonication was plotted in Figure 4.10 and 4.11. Increasing trends was shown as the time increased from 0 to 8 hours at temperature 40, 60 and 80 °C. At 50 °C the trend was different; increasing and decreasing patterns were shown from 0-7 hours and after 7 hours of extraction, respectively. The highest yield was recorded at temperature 50 °C after 7 hours of extraction with the value of 1.7056±0.1191 mg/g. Hence, at this temperature, necessary energy to disrupt the cell wall structure was provided which enhanced the extraction of intracellular desired compound. However, continuing the heating after it lead to degradation of some active compounds. The highest increment for the 20 % duty cycle was at 50 °C which is consistent with the result obtained at 10 % duty cycle. At 20 % duty cycle, the increment recorded compared to control sample was 1.70 fold.

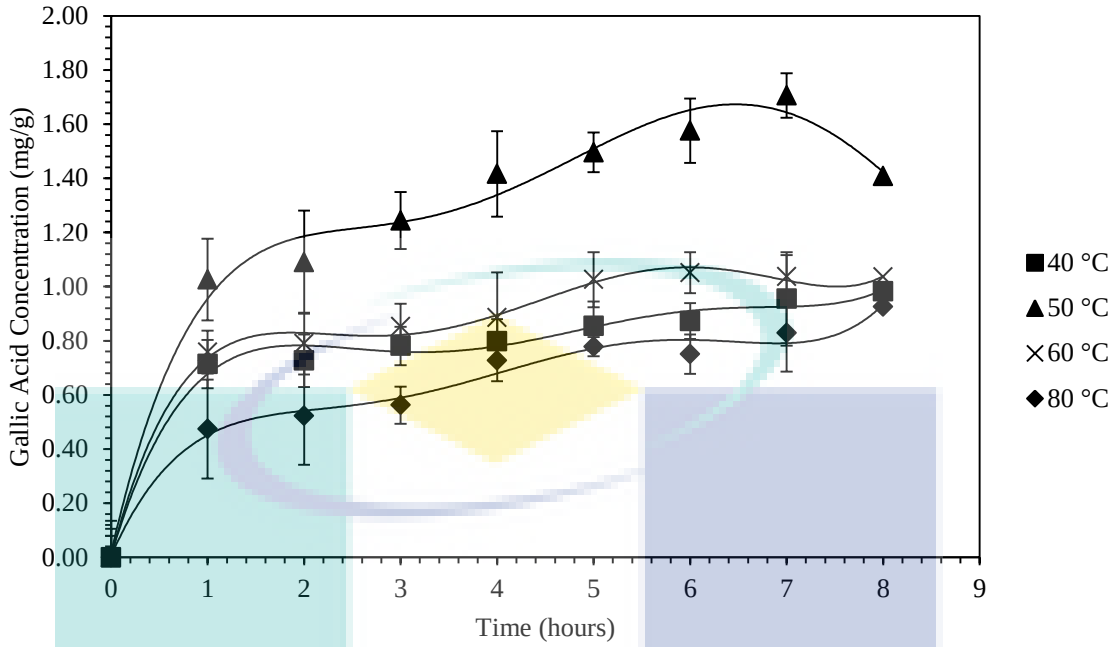


Figure 4.10 Effect of temperature 40, 50, 60, and 80 °C on the ultrasound-assisted extraction (UAE) with 20 % duty cycle in 8 hours

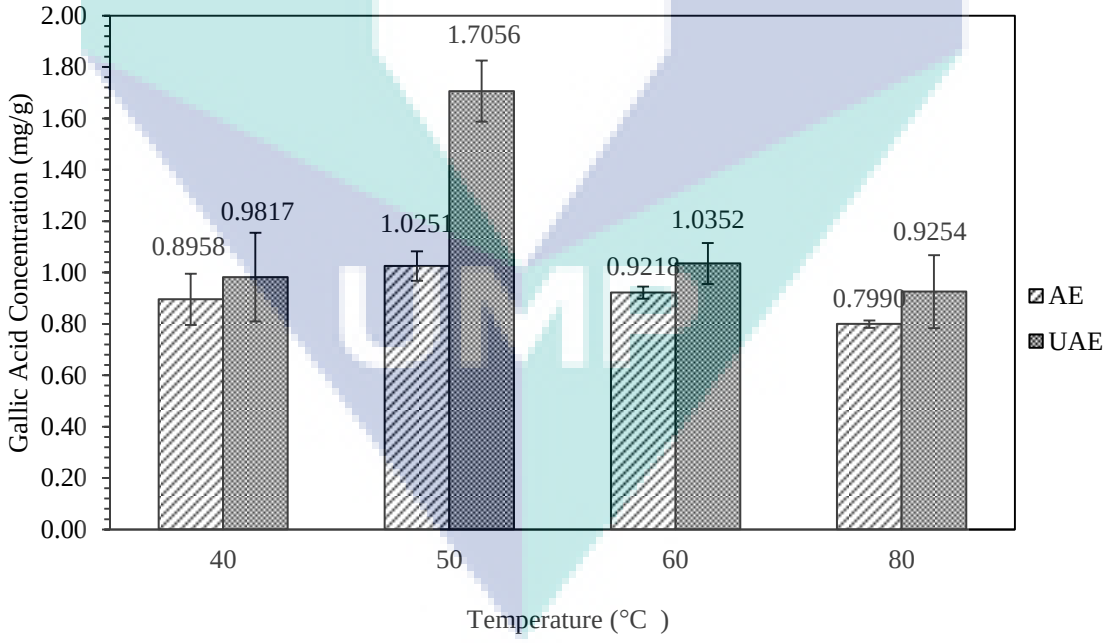


Figure 4.11 Comparison effect of conventional aqueous extraction (AE) and 20 % duty cycle of ultrasound-assisted extraction (UAE) at temperature 40, 50, 60, and 80 °C on the average gallic acid yield by 8 hours extraction and 1: 10 sample-to-water ratio

As expected, the highest yield obtained at duty cycle 40 % was at 50 °C which is consistent with the previous result. The result for 40 % duty cycle of sonication was plotted in Figure 4.12. The result is slightly different from the result obtained at 20 % duty cycle. The highest value of gallic acid obtained was shifted forward one hour (6th hour of extraction) compared to previous result (at 20 % duty cycle). At temperature 50 °C the trend was increasing from 0-6 hours and some of the active compounds started to degrade after 6 hours which lead to decreasing trend of gallic acid yield as shown in Figure 4.12. The highest yield recorded at 50 °C after 6 hours extraction was 1.8425±0.0772 mg/g which is also the highest increment for the 40 % duty cycle at 50°C. At 40 % duty cycle, the increment recorded compared to control sample was 2.00 fold. Therefore, sonication with duty cycle of 40 % gives better effect compared to 10 and 20 % duty cycle to extract gallic acid compound from *Labisia pumila* in sample-to-water ratio of 1:10 (g/mL). Sonication at 8.66 W/cm^2 and 40 % duty cycle provides more cell walls penetration and better mass transfer enhancement. For other temperature conditions, increasing trend was shown as the time increased from 0 to 8 hours.

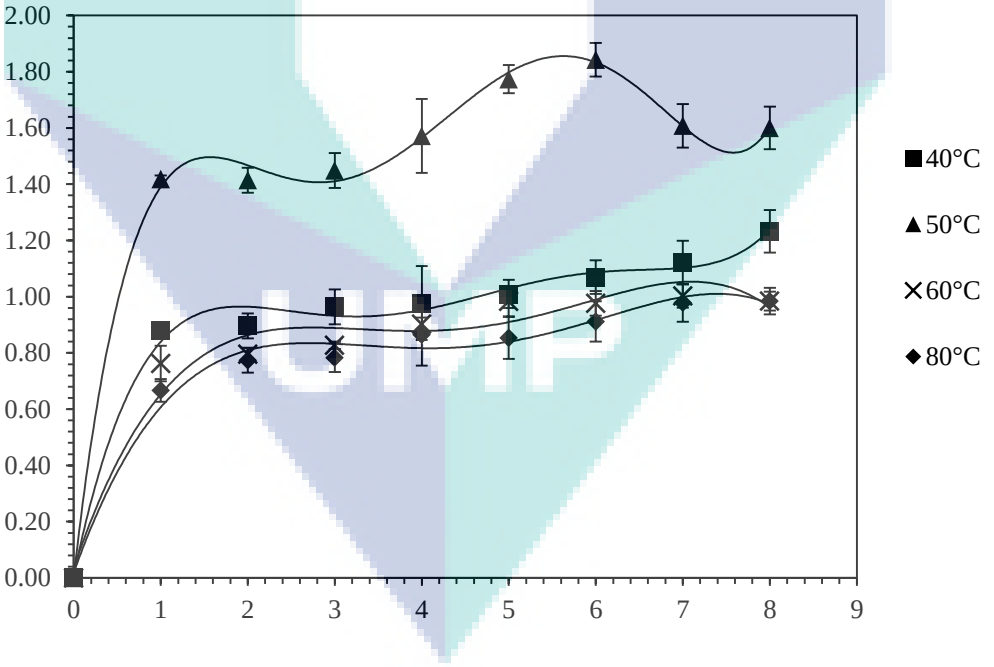


Figure 4.12 Effect of temperature 40, 50, 60, and 80 °C on the ultrasound-assisted extraction with 40 % duty cycle in 8 hours

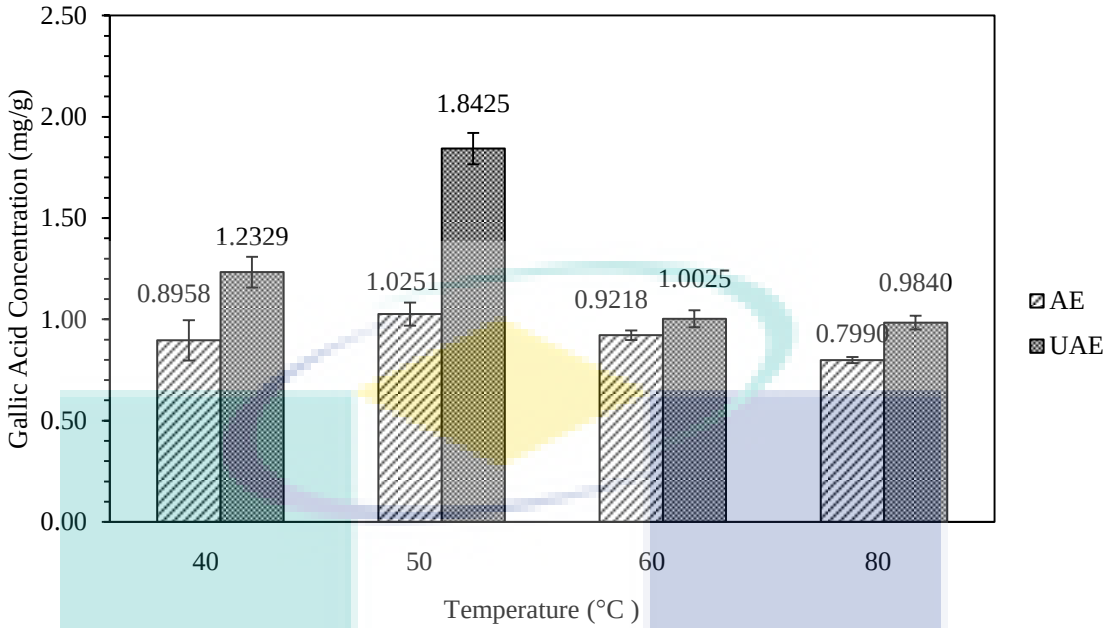


Figure 4.13 Comparison effect of conventional aqueous extraction (AE) and 40 % duty cycle of ultrasound-assisted extraction (UAE) at temperature 40, 50, 60, and 80 °C on the average gallic acid yield by 8 hours extraction and 1: 10 sample-to-water ratio

4.5 Comparison of aqueous extraction (AE) and ultrasound-assisted extraction (UAE)

In this section, the effect of 0 (control sample), 10, 20 and 40 % duty cycle sonication was compared at temperature 50 °C to determine the maximum gallic acid intracellular constituent obtained by UAE (Table 4.1). The sonication irradiation power was kept constant at 8.66 W/cm^2. The temperature condition of 50 °C was selected as it gave the highest value of gallic acid in each extract at 0(no sonication/control sample), 10, 20 and 40 % duty cycle (Table 4.1). This proved that, moderate temperature condition was the best for extraction of secondary metabolites especially gallic acid from *Labisia pumila*. As claimed by Palma et al.(2013) it is necessary to use less aggressive condition in extraction of natural product to avoid the degradation of the thermo sensitive compounds. As illustrated in Figure 4.14, the highest gallic acid yield obtained was at 40 % duty cycle after 6 hours extraction with the value of 1.8425 ± 0.1191 mg/g. The degradation of the compound occurred when the heating and sonication continued after the process achieved the maximum amount. The extraction at

0 and 10% duty cycle shows the increasing trend until the end of the process. This indicated that the desired compound still left in the sample but the energy and time provided is not enough to release the intracellular compound. In contrary, the result obtained at 20 and 40 % duty cycle shows that the extraction process achieved its maximum value at 7th and 6th hours. This proved that UAE can shorten the extraction process from more than 8 hours to 6 hours to achieve the maximum amount of gallic acid yield. Compared to AE, UAE improved 2.00 fold of gallic acid yield released after 6 hours of extraction at 50 °C. This finding is parallel to previous study on UAE from Yang et al.(2013) and Albu et al. (2004) which stated that, UAE can shorten the extraction time, and increase extraction efficiency and yields.

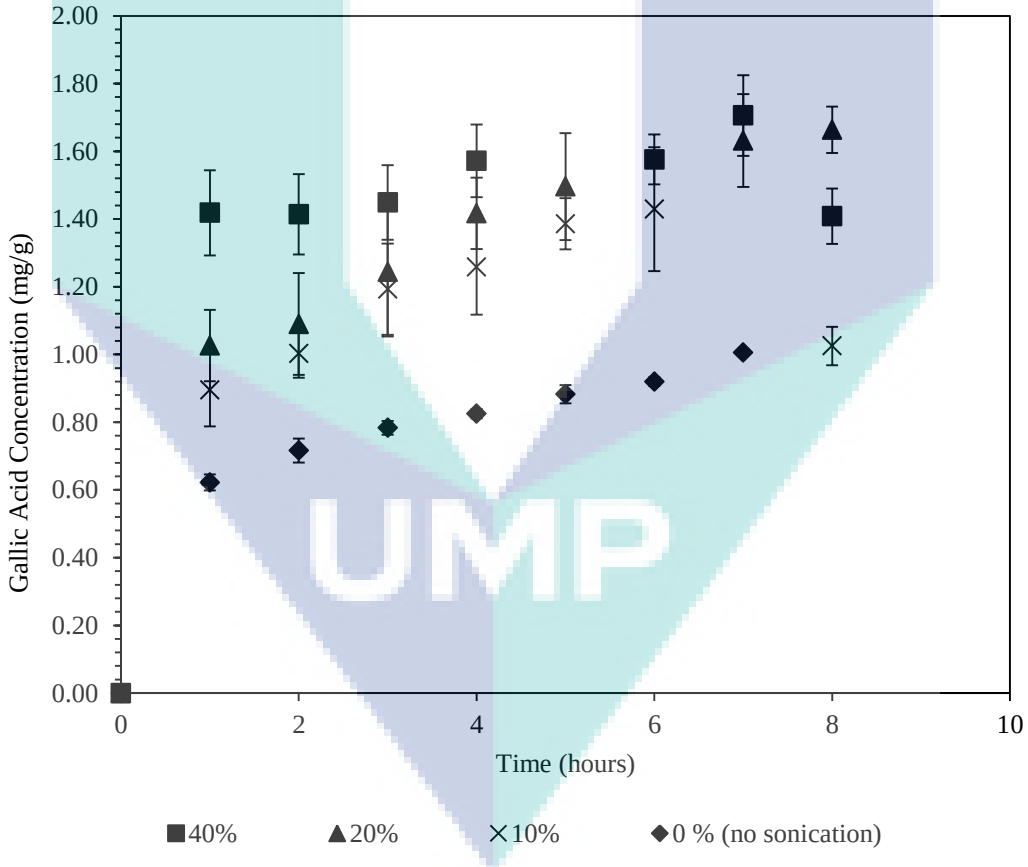


Figure 4.14 Comparison effect of 0(control sample), 10, 20 and 40 % duty cycle sonication at temperature 50 °C.

Table 4.1 Comparison of gallic acid yield from control sample (no sonication) and with sonication (10, 20 and 40 % duty cycle, Pir =8.66 W/cm^2)

Time	Control Sample (no sonication)	Sonication (10% duty cycle)[a]		Sonication (20% duty cycle)[a]		Sonication (40% duty cycle)[a]	
	Gallic Acid Concentration (mg/g)	Gallic Acid Concentration (mg/g)	Increment (Fold)	Gallic Acid Concentration (mg/g)	Increment (Fold)	Gallic Acid Concentration (mg/g)	Increment (Fold)
1	0.6221 ±0.0241	0.8948 ±0.1076	1.4384	1.0259 ±0.1055	1.6491	1.4183 ±0.1257	2.2798
2	0.7162 ±0.0357	1.0023 ±0.0713	1.3994	1.0901 ±0.1503	1.5220	1.4142 ±0.1186	1.9746
3	0.7834 ±0.0201	1.1926 ±0.1343	1.5224	1.2438 ±0.1904	1.5877	1.4490 ±0.1103	1.8497
4	0.8249 ±0.0134	1.2575 ±0.1405	1.5244	1.4164 ±0.1055	1.7170	1.5719 ±0.1072	1.9055
5	0.8829 ±0.0270	1.3857 ±0.0754	1.5695	1.4958 ±0.1580	1.6942	1.7738 ±0.0422	2.0091
6	0.9198 ±0.0124	1.4293 ±0.1828	1.5539	1.5760 ±0.0736	1.7134	1.8425 ±0.1191	2.0032
7	1.0056 ±0.0057	1.6317 ±0.1370	1.6225	1.7056 ±0.1191	1.6961	1.6077 ±0.1286	1.5987
8	1.0251 ±0.0569	1.6633 ±0.0688	1.6225	1.4080 ±0.0820	1.3735	1.6006 ±0.2134	1.5614

[a]Except for the control, the sonication power intensity was always 8.66 W/cm^2

4.6 Enzymatic extraction (EnE)

Study of the enzymatic extraction was conducted at 0.025, 0.05, 0.1, 0.2 and 0.3 g/L cellulase enzyme concentration. Then, the result obtained from enzymatic extraction (EnE) and control sample / aqueous extraction (AE) was compared (Figure 4.14). The control sample was not added with cellulase enzyme. The temperature and pH value was set at 50 °C, and 4.8, respectively and the extraction was carried out for 8 hours. The effect of different cellulase concentration on the amount of gallic acid yield was plotted in Figure 4.15. The highest yield of gallic acid recorded was 1.28565±0.1760 mg/g at the enzyme concentration of 0.05 g/L. Comparing this result to control sample, 1.2542 fold increments was recorded. Hence, the enzyme concentration of 0.05 g/L was selected to be applied in the next extraction methods. The increasing trend from 0 to 8 hours extraction for all the enzyme concentrations indicated that, the extraction of intracellular compound still not achieved the maximum value. For this method, the temperature was kept constant at 50 °C because the enzyme was most active at this temperature. Besides that, the glucose concentration determined in *Labisia pumila* extract was show a parallel result. From Dinitrosalicylic acid (DNS) essay by UV-Vis spectrophotometer 0.05 g/L of cellulse concentration shows the highest amount of glucose concentration (Figure 4.16). This proved that at the enzyme activity was highest at this concentration.

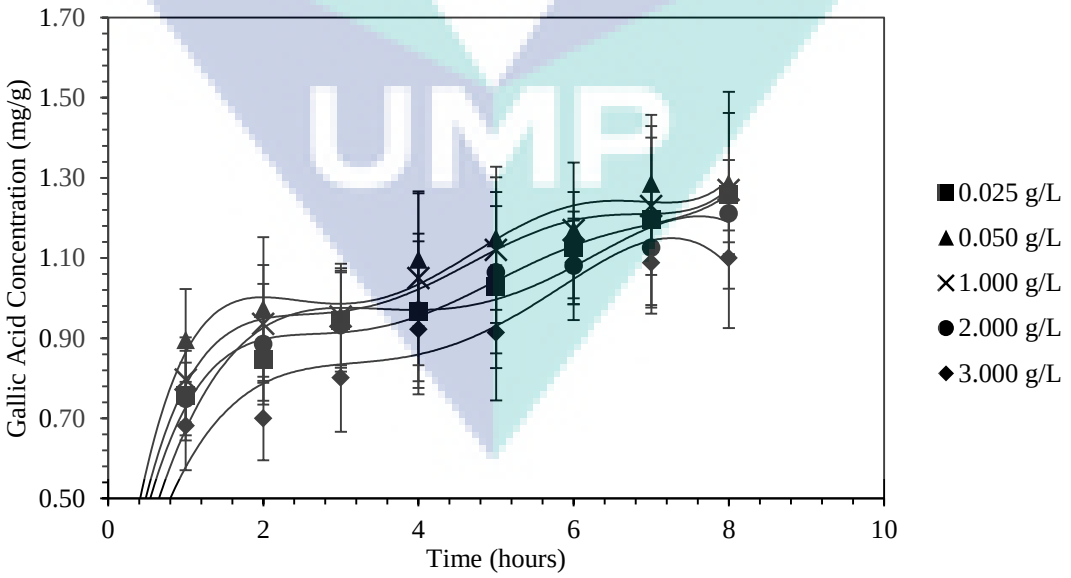


Figure 4.15 Effect of cellulase concentration 0.025, 0.05, 0.1, 0.2, 0.3 g/L on the enzymatic extraction at 50 °C for 8 hour

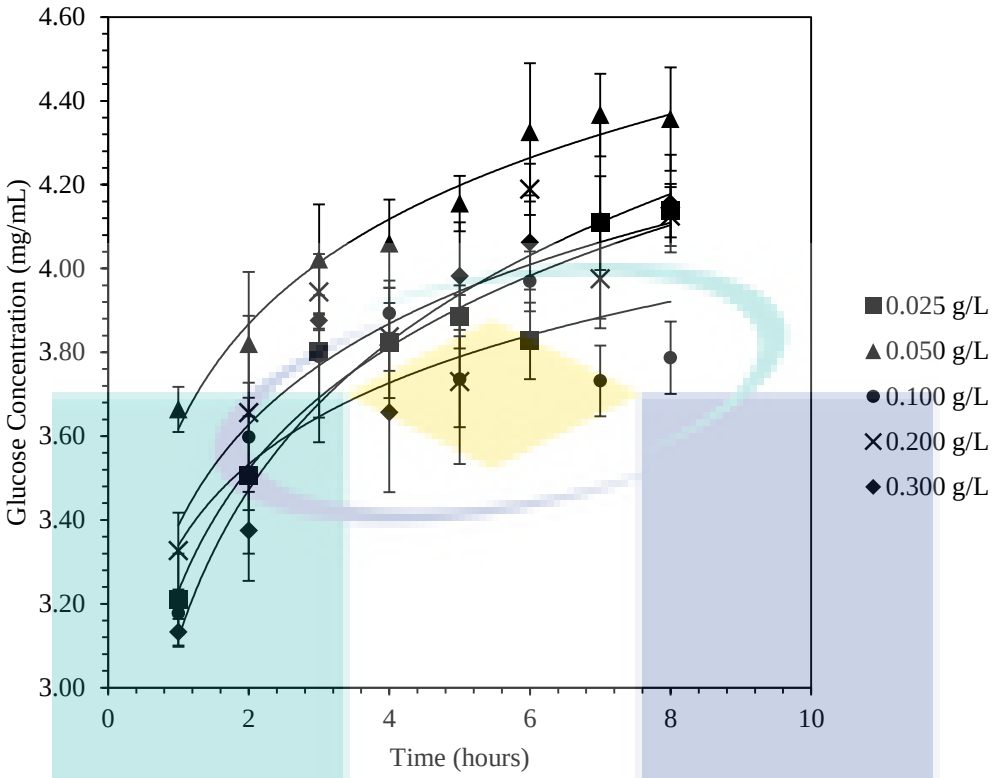


Figure 4.16 Glucose concentration determined in *Labisia pumila* extract with cellulase concentration 0.025, 0.05, 0.1, 0.2, 0.3 g/L on the enzymatic extraction at 50 °C for 8 hours

4.7 Comparison of aqueous extraction (AE) and enzymatic extraction (EnE)

The best result obtained from enzymatic extraction (EnE) was compared to the control sample (AE) at temperature 50 °C to determine the enhancement of enzyme activity towards the extraction process. As illustrated in Figure 4.17, the enhancement of gallic acid yield obtained from EnE method was shown from the 1st hour of extraction with 1.44 fold increment from 0.6221±0.0241 mg/g to 0.8950±0.1279 mg/g of gallic acid yield. Whereas, the highest gallic acid amount obtained from EnE method was 1.2857±0.1759 mg/g after 8 hours extraction. From Table 4.2, the enhancement of enzymatic activity was decreased from 1.44 fold at 1st hour to 1.30 fold at 8th hour of extraction. This indicated that the enzyme activity was decreased as the more cellulase was reacted with cellulose to loose the bonds in the cell wall. Enzymatic activity was facilitating cell matrix destruction; hence the more intracellular constituent was

released. Enzymatic hydrolysis enhanced the efficiency of extraction by attacking the cell matrix in the cell wall (Cho et. al.,2013).

Table 4.2 Comparison of gallic acid yield from control sample (no sonication) and with cellulase enzyme (0.05 g/L)

Time	0 mg/L (Control Sample)		0.05 mg/L Cellulse Concentration		
	Gallic Acid Concentration (mg/g)		Gallic Acid Concentration (mg/g)		Increament (Fold)
1	0.6221	±0.0241	0.8950	±0.1279	1.4387
2	0.7162	±0.0357	0.9727	±0.1791	1.3581
3	0.7834	±0.0201	0.9559	±0.1301	1.2203
4	0.8249	±0.0134	1.0953	±0.1657	1.3278
5	0.8829	±0.0270	1.1493	±0.1782	1.3017
6	0.9198	±0.0124	1.1690	±0.0289	1.2709
7	1.0056	±0.0057	1.2847	±0.1721	1.2775
8	1.0251	±0.0569	1.2857	±0.1759	1.2542

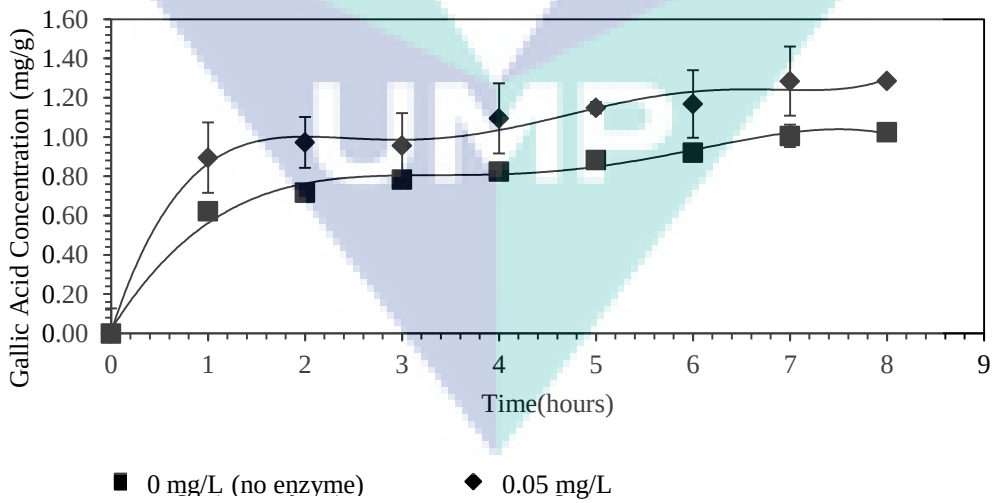


Figure 4.17 Comparison enzymatic extractions (EnE) at 0.05 g/L cellulase concentration with aqueous extraction (AE) 50 °C for 8 hours

4.8 Ultrasound-assisted enzymatic extraction (UAEnE)

The best condition obtained from AE, UAE and EnE was combined for UAEnE method. From all previous method indicated that 50 °C is the most suitable to use in this part since it was proven to gives the highest gallic acid extracted value compared to other temperature for AE and UAE method. Furthermore, the EnE method also shows the positive result, which is the enzymatic hydrolysis improved the extraction process at the same temperature. For sonication regimens, 40 % duty cycle at 8.66 W/cm^2 was selected to apply in this method. From previous result, the highest gallic acid extracted from *Labisia pumila* by UAE method was 1.8425±0.1191 mg/g with 2.0 fold increment compared to AE method. Whereas, EnE improved the extraction process achievement by 1.3 fold. Figure 4.18 shows the result obtained when the UAE was combined with EnE. The increasing trend was indicated as time increasing from 1 to 7 hour. When the extraction process was prolonged to 8 hours of some of the compounds started to degrade. The highest gallic acid amount obtained from UAEnE was 2.9287±0.4060 mg/g after 7 hours extraction with 2.91 fold increment compared to AE result (Table 4.3). The enhancement achieved in UAEnE method was higher than UAE and EnE because the sonication promoted the enzyme hydrolysis by facilitating the hydration and swelling which lead to pore enlargement of the cell wall and enhanced the diffusion, reaction and mass transfer in the process (Vinatoru, 2001**)**.

Table 4.3 Comparison of gallic acid yield extracted from Aqueous Extraction(AE) and Ultrasound-assisted Enzymatic Extraction (UAEnE)

Time	**Aqueous Extraction (AE)**	**Ultrasound-assisted Enzymatic Extraction (UAEnE)**	
	Gallic Acid Concentration (mg/g)	**Gallic Acid Concentration (mg/g)**	**Increament (Fold)**
1	0.6221 ±0.0241	1.8641 ±0.1754	2.9964
2	0.7162 ±0.0357	2.0278 ±0.2348	2.8314
3	0.7834 ±0.0201	2.1586 ±0.1270	2.7554
4	0.8249 ±0.0134	2.2055 ±0.1654	2.6736
5	0.8829 ±0.0270	2.6135 ±0.1908	2.9601
6	0.9198 ±0.0124	2.8005 ±0.3204	3.0446

Table 4.3 Continued

Time	Aqueous Extraction (AE)		Ultrasound-assisted Enzymatic Extraction (UAEnE)		
	Gallic Acid Concentration (mg/g)		Gallic Acid Concentration (mg/g)		Increament (Fold)
7	1.0056	±0.0057	2.9287	±0.4060	2.9122
8	1.0251	±0.0569	2.8668	±0.1902	2.7966

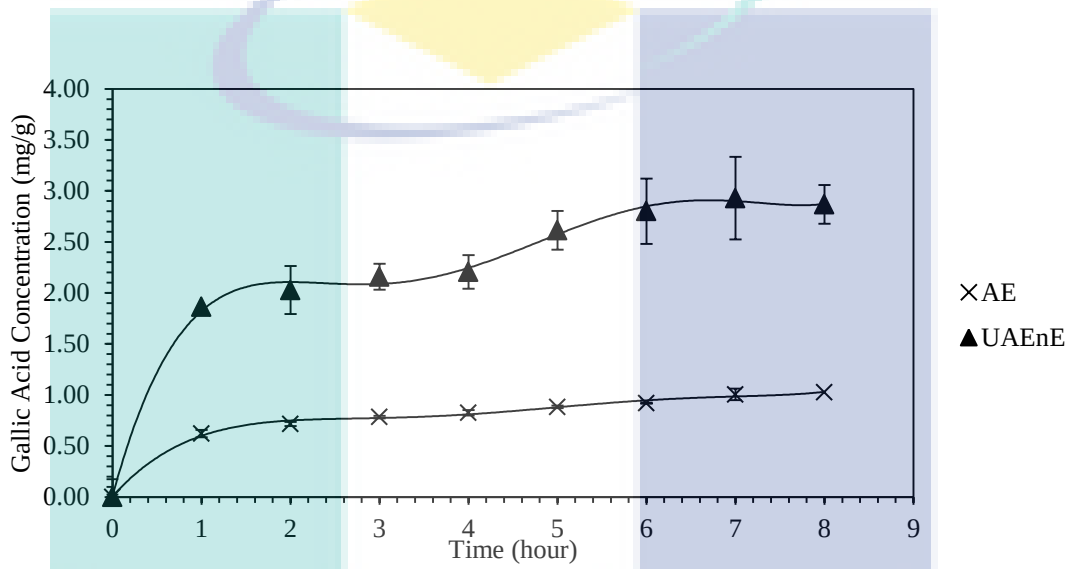


Figure 4.18 Ultrasound-assisted enzymatic extractions (UAEnE) at 0.05 g/L cellulase concentration and 40 % duty cycle for 8 hours

In this final section, conclusion was drawn concerning the several factors affecting the performance of extraction in 4 different methods. In general, UAE, EnE and UAEnE enhanced the extraction performance by improving and facilitating the mass transfer. From the experimental data obtained, the application of ultrasound and enzyme were succeeded in improving the extraction process. However, the enhancement of the gallic acid yield achieved from the ultrasound and enzyme combination are really impressive. Figure 4.19 summarized all the result obtained in this study. Based on the results of this study, the best condition for each extraction methods were concluded in Table 4.4. Temperature 50 °C dominates at each method and hour of extraction process.

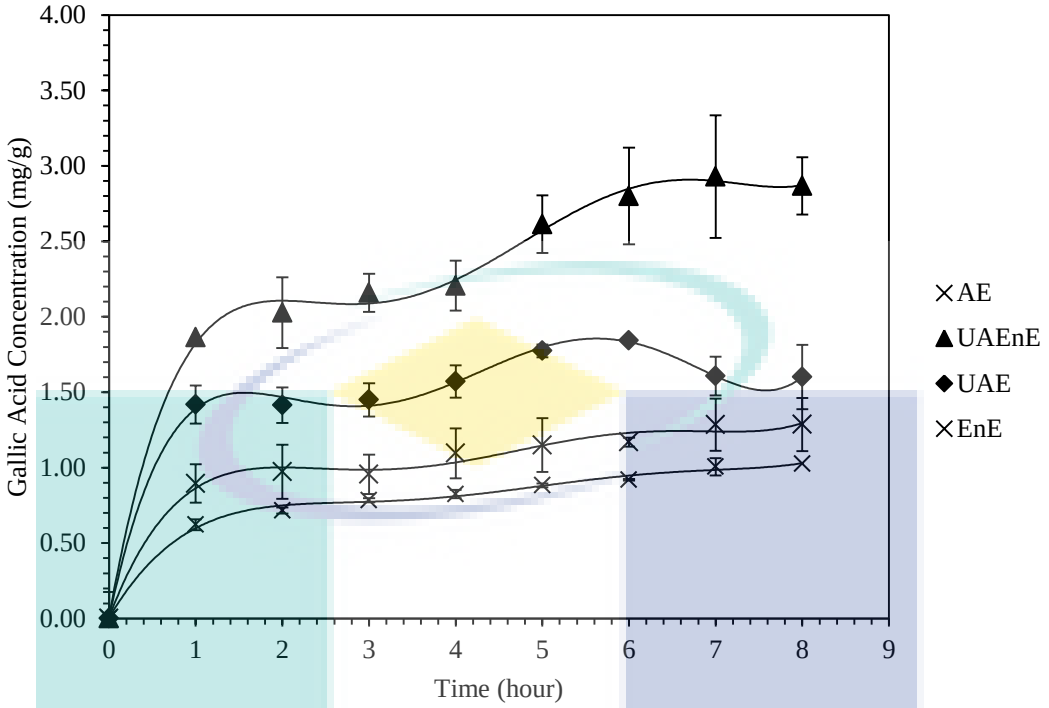


Figure 4.19 Overall comparison results for AE, UAE, EnE and UAEnE at temperature 50 °C and 1:10 Sample-to-water ratio

Table 4.4 Summary best conditions for AE, UAE, EnE and UAEnE methods

Extraction Method	**AU**	**UAE**	**EnE**	**UAEnE**
Sample-to-water ratio (g/mL)	1:10	1:10	1:10	1:10
Temperature (°C)	50	50	50	50
Sonication duty cycle (%)	-	40	-	40
Enzyme concentration (g/l)	-	-	0.05	0.05

4.9 Identification of gallic acid in *Labisia pumila* extracts

Liquid Chromatography Mass Spectrophotometer-Quadrupole Time-of-Flight (LCMS-QTOF) and developed databases system Plant Metabolic Network (PMN) and Metabolite database (METLIN) were used to identify the gallic acid in aqueous extraction (AE) and integrated ultrasound-assisted enzymatic extraction method (UAEnE) of *Labisia pumila*. Figure 4.20 and 4.21 shows the mass spectrum of extracted samples. Selected metabolites present were tabulated in Table 4.5. The mass spectrum

obtained from the analysis was compared with mass spectrum of gallic acid which was available in Metlin Database, the pattern of spectrum was similar as shown in Figure 4.20 and 4.21. Figure 4.20 was the mass spectrum of gallic acid at 0 Volt collision energy for aqueous extraction, whereas Figure 4.21 at for integrated ultrasound-assisted enzymatic extraction method (UAEnE). This indicated that the gallic acid compound presence in the *Labisia pumila* extract is confirmed refer to Figure 4.20. Furthemore, as showed in Figure 4.21, gallic acid was not affected nor denatured with application of enzyme and low intensity of sonication. As indicated from HPLC-DAD analysis, yield of gallic acid was improved with both enzyme and sonication applied. Moreover, after checking the mass spectral characteristics and identity at PMN and Metlin Database, 5 metabolites detected in the extrac were tabulated in Table 4.5. However, thus study just focused on the gallic acid for the marker compound of *Labisia pumila* (Malaysian Standard, 2013). From the analysis it showed that the gallic acid metabolites were identified at retention time of 1.146 with the mass-to-charge ratio of 169.014 m/z.

Table 4.5 Mass spectral characteristics and identity of some important metabolites present in *Labisia pumila* extract

tR (min)	**Compound**	**$[M-H]^-$ (m/z)**	**tR (min)**
1.146	Gallic Acid	169.014	1.146
1.486	Syringic Acid	197.0457	1.486
1.764	Vanillic Acid	167.0348	1.764

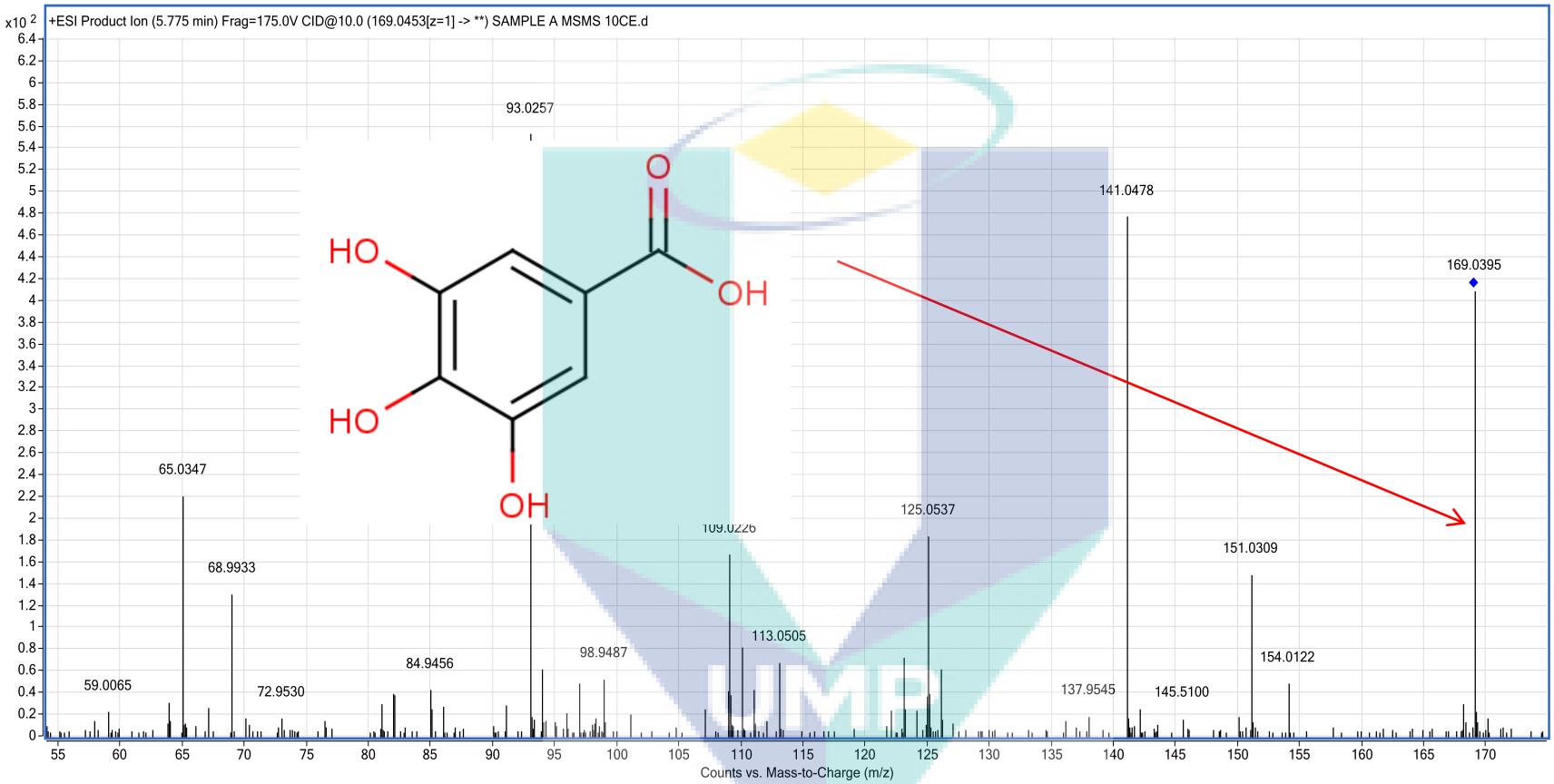


Figure 4.20 Mass spectral characteristics and identity of phenolic present in extract of *Labisia pumila* analyzed by LCMS-QTOF at 0 Volt collision energy for aqeous extraction method (AE)

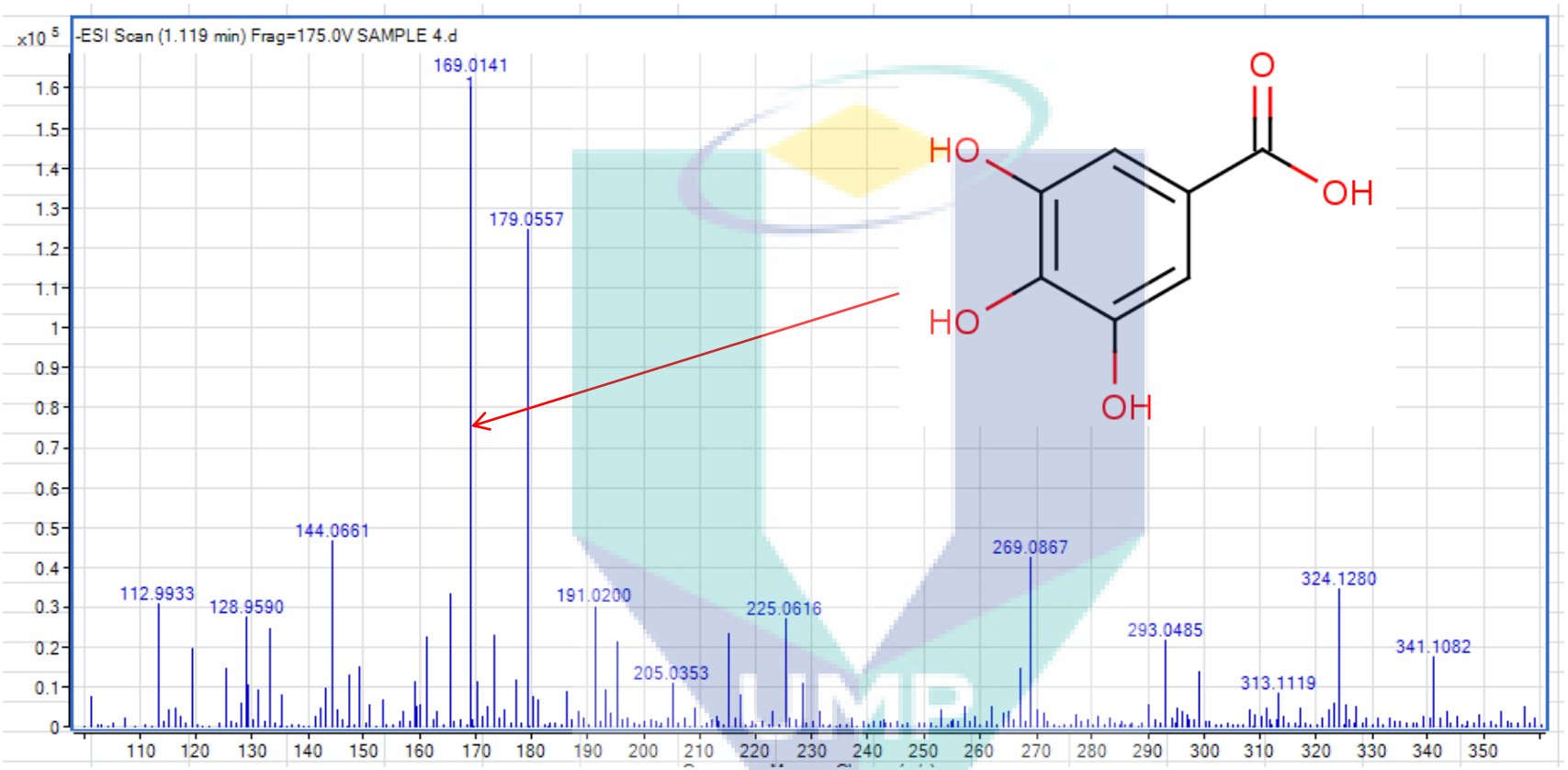


Figure 4.21 Mass spectral characteristics and identity of phenolic present in extract of *Labisia pumila* analyzed by LCMS-QTOF at 0 Volt collision energy for integrated ultrasound-assisted enzymatic extraction method (UAEnE)

4.10 The physical effect on the *Labisia pumila* extracts

Field emission scanning electron microscopy (FESEM) was carried out on the ultrasonic-assisted extract and aqueous extract after the extraction was done. FESEM is carried out to study the morphological surface structure of *Labisia pumila* sample after the extraction. It is also to study the effect of acoustic cavitation of ultrasound and the application of enzyme on cell wall structure. Table 4.6 shows FESEM observation of *Labisia pumila* leaves surface structure on aqueous extraction (AE), ultrasound-assisted extraction (UAE), enzymatic extraction (EnE) and ultrasound-assisted enzymatic extraction (UAEnE) at x3,000 and x10,000 magnifications.

At both x3000 and x10000 magnifications, the surface structure of sample for AE shows the less damaged compared to all other method of extraction. Smooth and undamaged surface can be observed for AE method. For UAE, exfoliated surface can be seen and the surface damaged was more than AE. This results from the effect of the violent shock produce during the collapsing of microbubble from ultrasound application during the extraction process. Ultrasound enhance the extraction process by improving the penetration of the solvent to the plant structure, hence the intracellular compound quickly diffuse in the solvent (Wang et al., 2008). From the previous study, extraction of total carbohydrates from *Stevia rebaudiana Bertoni* was improved by application of ultrasound (Liu et al., 2010).

Besides, refer to sample with enzymatic extraction for both magnifications, the sample structure was more wrinkled, which showed that more cell wall was destructed by the enzyme activity. As stated by Doi & Kosugi (2004) cellulase converts the cellulosic biomass into sugar which result an improvement in the production of organic acid. The intergration of sonication and enzyme activity on the extraction process of *Labisia pumila* give totally destructed surface. This showed that both sonication and enzymatic activity helps the extraction process with produce more porous surface, hence easier to the gallic acid diffuse in the water. As UAE was improving the enlargement the pore in the cell wall, this enhanced the mass transfer during the extraction. From the morhphological study, integrated ultrasound-assisted enzymatic extraction gives the better impact on the surface of the dried extracted *Labisia pumila*.

Table 4.6 FESEM observation of *Labisia pumila* leaves surface structure on aqueous extraction (AE), ultrasound-assisted extraction (UAE), enzymatic extraction (EnE) and ultrasound-assisted enzymatic extraction (UAEnE) at x3,000 and x10,000 magnifications

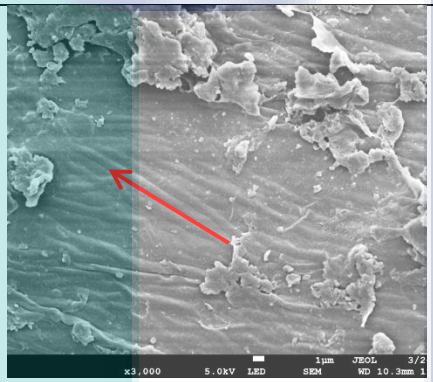
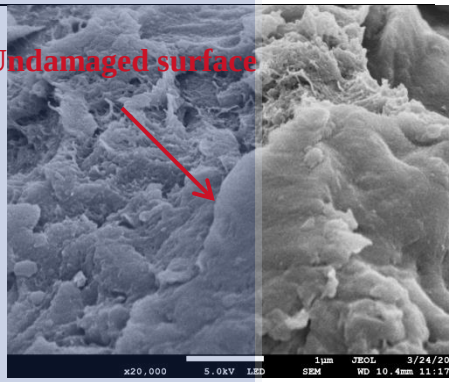
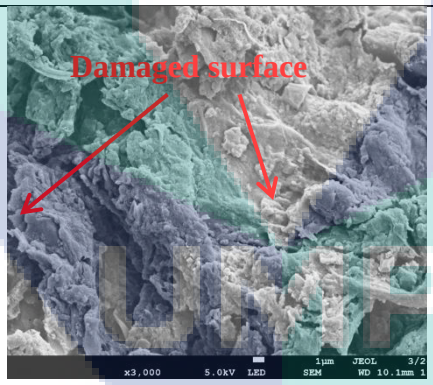
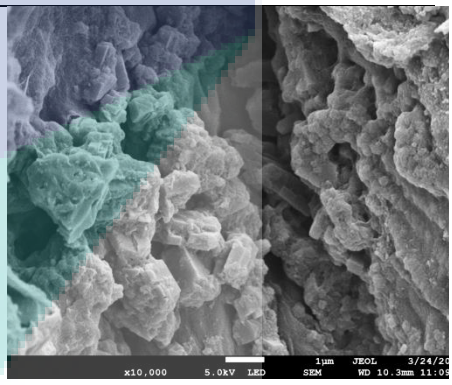
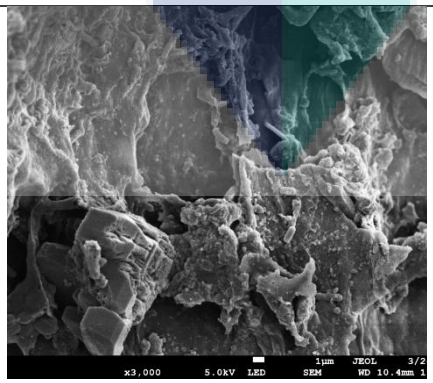
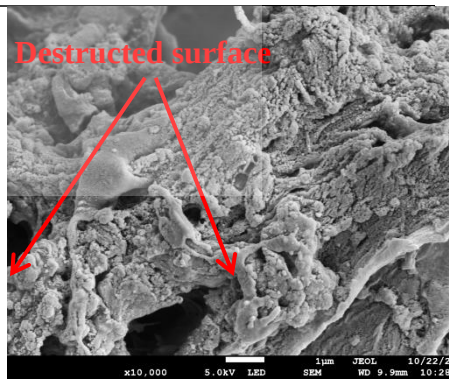
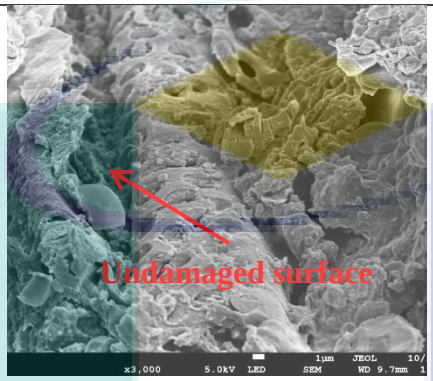
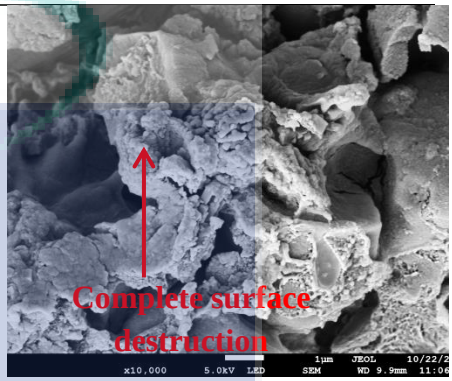
Extraction Method	**Magnification**	
	3000	**10000**
Aqueous Extraction (AE)		
Ultrasound-assisted Extraction (UAE)		
Enzymatic Extraction (EnE)		

Table 4.22 Continued

Extraction Method	Magnification	
	3000	10000
Aqueous Extraction (AE)		

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, the effect of sonication, enzyme and also integration of both sonication and enzyme methods were studied. All extraction methods successfully extracted the target compound gallic acid from *Labisia pumila* and thus the objectives of this work were achieved. Following highlights the specific conclusions of the study:

i. The integrated Ultrasonic-Assisted Enzymes (UAEnE) as a single extraction unit for gallic acid from *Labisia Pumila* (Kacip fatimah) was a complicated work since many parameters were monitored. The highest gallic acid amount obtained from this method was at 40 % duty cycle and 0.05 g/L cellulase concentration with 2.9287±0.4060 mg/g gallic acid extracted after 7 hours of extraction. This recorded 2.91 fold increment compared to AE result. In the AE process, after 7 hours extraction process the amount of gallic acid extracted was 1.0056±0057 mg/g. The extraction yield may have improved because of sonication that produced cavitation bubbles and enhanced the cellulase activity besides the cellulase activity enhanced the release of gallic acid from plant matrix.

ii. Intermittent sonication with ultrasound power (11 W, 8.66 W/cm^2) at duty cycle of 40 % effectively enhanced the extraction process of *Labisia pumila* relative to control and yielded optimum gallic acid of 1.8425±0.1191 mg/g after 6 hours extraction. Compared to the control with the same process time, 2.0 fold improvements were recorded in UAE method compared to the aqueous extraction. In enzymatic extraction (EnE), the highest yield recorded was at the enzyme concentration of 0.05 g/L with value 1.28565±0.1760 mg/g of gallic acid (1.2542 fold improvement compare to aqueous extraction) at 50 FºC for 8

h. From an aqueous extraction method, sample-to-solvent ratio of 1:10 was the best ratio and 50 ºC was the optimum temperature for obtaining optimum yield (1.0251±0.0569 mg/g) of gallic acid extraction from *Labisia pumila* after 8 hours of extraction.

iii. Gallic acid metabolites quantified from *Labisia pumila* was confirmed using Accurate-Mass Quadruple Time-of-Flight (Q-TOF) LC/MS , developed databases system Plant Metabolic Network (PMN) and Metabolite database (METLIN)). The gallic acid metabolites was found at 1.146 retention time with 169.014 mass-to-charge ratio, m/z.

5.2 Recommendations

Sonication and enzyme with suitable regimens and concentration has a potential in improving extraction process of intracellular constituent. Two recommendations for the better exploration for both conditions are as follows:

i. This research should be extend with other sonication regimen such as power and intensity need to be studied as they might affect the process; hence the study on varying ultrasound power and intensity in the extraction of *Labisia pumila* can have better improvements in yield production.

ii. Since, there are many other active compound were present in the extract, hence for purpose of further research, identification and isolation of all active compound surely will give a huge contribution in this research area.

For commercialization purpose, an ultrasonic equipment extractor with control panel to control the temperature and sonication intensity should be fabricate so then can produce more gallic acid extract.

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APPENDIX 1
Sonication Intensity Calculation

$$I = \frac{P}{A}$$

Where

A was the area of the sonotrode tip

P was the ultrasound power.

Mobile phase concentration calculation for High Performance Liquid Chromatography Analysis

$$c1\ v1 = c2\ v2$$

Where

c1 was the initial concentration of the solution
v1 was the initial volume
c2 was the final concentration of the solution
v2 was the initial volume

Gallic acid yield concentration calculation

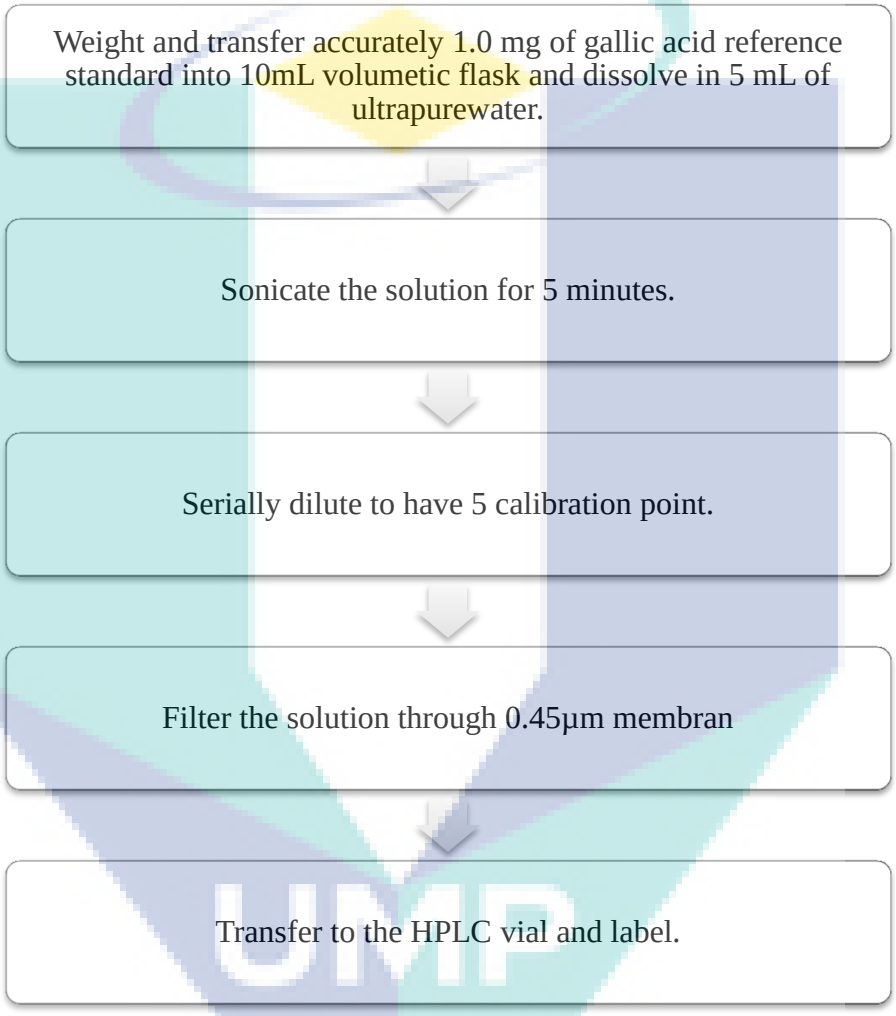
Sample A (mg of gallic acid / kg of dry sample) =

$$\frac{(concentration\ got\ from\ HPLC\ analysis, ppm)x\ (volume\ of\ solvent\ during\ extraction, l)x(dilution\ factor)}{mass\ of\ sample\ use\ during\ extraction, kg}$$

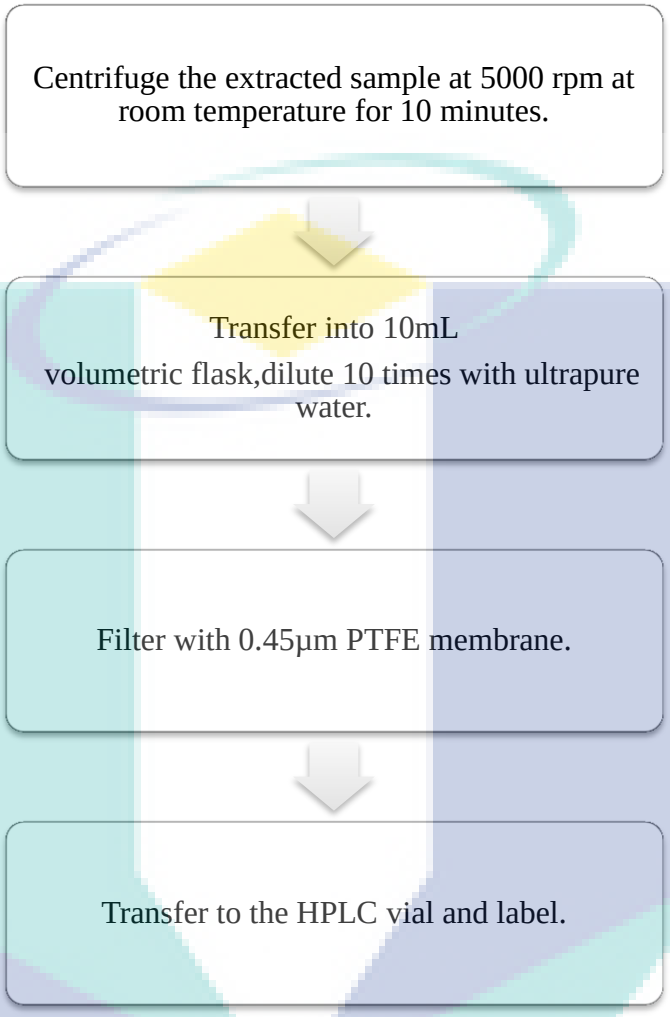
APPENDIX 2

High Performance Liquid Chromatography (HPLC) Analysis Procedure

A Preparation of gallic acid standard



B Preparation of Sample

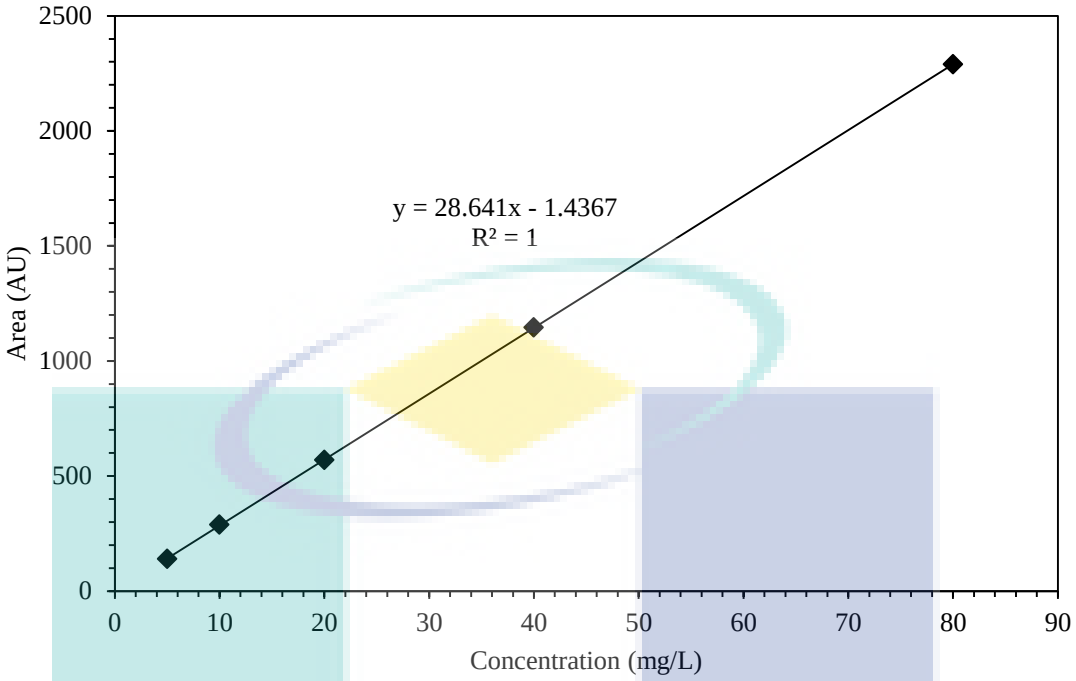


C Method of Gallic acid Analysis

Column	C18 column, 4.6mm x 250mm ,5µm particle size or equivalent
Mobile phase	A : Acetonitrile B : 3% Phosphoric buffer water
Analysis mode	Isocratic (Solvent system A/B=10/90)
Flowrate	1mL/min
Wavelength	UV at 270nm
Retention time	4-7 minutes
Run time	10 minutes

APPENDIX 3
HPLC Calibration Curve

Concentration (mg/L)	Area (AU)
5	139.3883
10	288.401
20	569.2983
40	1145.801
80	2289.293


2500
2000
1500
1000
500
0
Area (AU)
y = 28.641x - 1.4367
R² = 1
0
10
20
30
40
50
60
70
80
90
Concentration (mg/L)

APPENDIX 4

Baseline Curve / Aqueous Extraction (AE)

A Effect of sample-to-solvent ratio

Sample-to-Solvent Ratio	Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)			Mean	Standard Deviation
		A	B	C			A	B	C	A	B	C	A	B	C		
1:06	0	0.00	0.00	0.00	0.042857	0.2571	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	217.38	222.92	213.45	0.042857	0.2571	7.6008	7.7945	7.4632	456.0474	467.6677	447.7902	0.4560	0.4677	0.4478	0.4572	0.0100
	2	292.64	292.21	265.52	0.042857	0.2571	10.2322	10.2172	9.2838	613.9344	613.0309	557.0302	0.6139	0.6130	0.5570	0.5947	0.0326
	3	307.21	333.74	293.68	0.042857	0.2571	10.7415	11.6693	10.2684	644.4878	700.1590	616.1052	0.6445	0.7002	0.6161	0.6536	0.0428
	4	351.15	370.83	345.05	0.042857	0.2571	12.2781	12.9660	12.0647	736.6850	777.9600	723.8817	0.7367	0.7780	0.7239	0.7462	0.0283
	5	366.36	383.33	356.39	0.042857	0.2571	12.8096	13.4030	12.4611	768.5777	804.1805	747.6645	0.7686	0.8042	0.7477	0.7735	0.0286
	6	368.70	395.47	360.36	0.042857	0.2571	12.8916	13.8277	12.5998	773.4963	829.6643	755.9902	0.7735	0.8297	0.7560	0.7864	0.0385
	7	389.49	423.70	376.94	0.042857	0.2571	13.6186	14.8146	13.1797	817.1167	888.8736	790.7833	0.8171	0.8889	0.7908	0.8323	0.0508
	8	402.58	435.57	402.69	0.042857	0.2571	14.0763	15.2297	14.0800	844.5800	913.7845	844.8007	0.8446	0.9138	0.8448	0.8677	0.0399
1:08	0	0.00	0.00	0.00	0.033333	0.2667	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	190.50	177.85	184.91	0.033333	0.2667	6.6610	6.2186	6.4652	532.8774	497.4903	517.2194	0.5329	0.4975	0.5172	0.5159	0.0177
	2	214.34	221.48	222.79	0.033333	0.2667	7.4944	7.7442	7.7900	599.5538	619.5355	623.1998	0.5996	0.6195	0.6232	0.6141	0.0127
	3	247.20	234.85	246.98	0.033333	0.2667	8.6433	8.2115	8.6358	691.4687	656.9198	690.8635	0.6915	0.6569	0.6909	0.6798	0.0198
	4	272.12	270.81	276.34	0.033333	0.2667	9.5147	9.4689	9.6622	761.1749	757.5153	772.9808	0.7612	0.7575	0.7730	0.7639	0.0081
	5	289.57	295.60	275.60	0.033333	0.2667	10.1250	10.3357	9.6364	810.0014	826.8605	770.9151	0.8100	0.8269	0.7709	0.8026	0.0287

	6	302.34	301.23	284.88	0.033333	0.2667	10.5714	10.5326	9.9609	845.7127	842.6105	796.8726	0.8457	0.8426	0.7969	0.8284	0.0273
	7	303.77	303.65	318.69	0.033333	0.2667	10.6214	10.6172	11.1432	849.7153	849.3769	891.4562	0.8497	0.8494	0.8915	0.8635	0.0242
	8	309.55	309.23	327.71	0.033333	0.2667	10.8235	10.8121	11.4585	865.8789	864.9735	916.6794	0.8659	0.8650	0.9167	0.8825	0.0296
1:10	0	0.00	0.00	0.00	0.027273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	164.41	166.96	159.93	0.027273	0.2727	5.7486	5.8377	5.5920	574.8565	583.7725	559.1952	0.5749	0.5838	0.5592	0.5726	0.0124
	2	186.83	182.47	180.53	0.027273	0.2727	6.5326	6.3799	6.3121	653.2583	637.9904	631.2107	0.6533	0.6380	0.6312	0.6408	0.0113
	3	199.61	212.53	193.05	0.027273	0.2727	6.9794	7.4312	6.7501	697.9385	743.1153	675.0087	0.6979	0.7431	0.6750	0.7054	0.0347
	4	218.63	224.48	216.41	0.027273	0.2727	7.6445	7.8490	7.5669	764.4545	784.8963	756.6859	0.7645	0.7849	0.7567	0.7687	0.0146
	5	227.44	225.53	223.69	0.027273	0.2727	7.9525	7.8857	7.8213	795.2532	788.5741	782.1348	0.7953	0.7886	0.7821	0.7887	0.0066
	6	238.29	247.57	226.59	0.027273	0.2727	8.3319	8.6564	7.9227	833.1859	865.6395	792.2744	0.8332	0.8656	0.7923	0.8304	0.0368
	7	245.11	255.95	254.52	0.027273	0.2727	8.5702	8.9494	8.8994	857.0199	894.9434	889.9448	0.8570	0.8949	0.8899	0.8806	0.0206
	8	262.99	284.22	270.47	0.027273	0.2727	9.1954	9.9378	9.4569	919.5371	993.7820	945.6934	0.9195	0.9938	0.9457	0.9530	0.0377
1:12	0	0.00	0.00	0.00	0.023077	0.2769	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	132.89	130.40	132.40	0.023077	0.2769	4.6465	4.5596	4.6293	557.5852	547.1532	555.5142	0.5576	0.5472	0.5555	0.5534	0.0055
	2	144.57	153.60	141.76	0.023077	0.2769	5.0548	5.3707	4.9568	606.5766	644.4843	594.8179	0.6066	0.6445	0.5948	0.6153	0.0260
	3	164.45	170.20	166.12	0.023077	0.2769	5.7498	5.9512	5.8084	689.9806	714.1423	697.0045	0.6900	0.7141	0.6970	0.7004	0.0124
	4	181.93	180.99	173.04	0.023077	0.2769	6.3610	6.3284	6.0504	763.3233	759.4087	726.0427	0.7633	0.7594	0.7260	0.7496	0.0205
	5	187.53	188.98	183.01	0.023077	0.2769	6.5570	6.6077	6.3990	786.8451	792.9246	767.8771	0.7868	0.7929	0.7679	0.7825	0.0131
	6	199.20	191.28	201.83	0.023077	0.2769	6.9649	6.6881	7.0569	835.7873	802.5767	846.8260	0.8358	0.8026	0.8468	0.8284	0.0230
	7	215.51	198.09	208.16	0.023077	0.2769	7.5352	6.9264	7.2785	904.2208	831.1681	873.4169	0.9042	0.8312	0.8734	0.8696	0.0367
	8	220.85	224.17	220.74	0.023077	0.2769	7.7219	7.8382	7.7183	926.6288	940.5898	926.1977	0.9266	0.9406	0.9262	0.9311	0.0082

B Effect of temperature

Temperature (ºC)	Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)			Mean	Standard Deviation
		A	B	C			A	B	C	A	B	C	A	B	C		
40	0	0.0000	0.0000	0.0000	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	135.1953	137.3839	139.6822	0.0273	0.2727	4.7271	4.8036	4.8840	472.7112	480.3639	488.3999	0.4727	0.4804	0.4884	0.4805	0.0078
	2	171.4050	153.6553	162.0632	0.0273	0.2727	5.9932	5.3726	5.6665	599.3189	537.2568	566.6552	0.5993	0.5373	0.5667	0.5677	0.0310
	3	180.0635	178.7074	177.0982	0.0273	0.2727	6.2959	6.2485	6.1922	629.5932	624.8518	619.2253	0.6296	0.6249	0.6192	0.6246	0.0052
	4	192.2035	187.8692	202.1660	0.0273	0.2727	6.7204	6.5689	7.0687	672.0410	656.8859	706.8749	0.6720	0.6569	0.7069	0.6786	0.0256
	5	224.7155	210.7976	217.4094	0.0273	0.2727	7.8572	7.3705	7.6017	785.7192	737.0553	760.1736	0.7857	0.7371	0.7602	0.7610	0.0243
	6	202.6230	229.3572	207.9591	0.0273	0.2727	7.0847	8.0195	7.2713	708.4728	801.9492	727.1305	0.7085	0.8019	0.7271	0.8019	0.0495
	7	253.2007	278.5929	281.3630	0.0273	0.2727	8.8532	9.7410	9.8379	885.3182	974.1020	983.7878	0.8853	0.9741	0.9838	0.9477	0.0543
	8	235.6405	288.6923	244.2299	0.0273	0.2727	8.2392	10.0941	8.5395	823.9186	1009.4147	853.9515	0.8239	1.0094	0.8540	0.8958	0.0996
50	0	0.0000	0.0000	0.0000	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	181.0590	182.6820	170.0347	0.0273	0.2727	6.3307	6.3875	5.9453	633.0742	638.7488	594.5274	0.6331	0.6387	0.5945	0.6221	0.0241
	2	214.1398	206.4353	193.9284	0.0273	0.2727	7.4874	7.2180	6.7807	748.7415	721.8025	678.0722	0.7487	0.7218	0.6781	0.7162	0.0357
	3	220.4925	220.9708	230.6755	0.0273	0.2727	7.7095	7.7263	8.0656	770.9537	772.6260	806.5585	0.7710	0.7726	0.8066	0.7834	0.0201
	4	236.5440	239.4276	231.8092	0.0273	0.2727	8.2708	8.3716	8.1052	827.0778	837.1604	810.5227	0.8271	0.8372	0.8105	0.8249	0.0134
	5	261.2314	246.5346	249.7734	0.0273	0.2727	9.1340	8.6201	8.7333	913.3975	862.0099	873.3346	0.9134	0.8620	0.8733	0.8829	0.0270
	6	262.6618	266.7848	259.7445	0.0273	0.2727	9.1840	9.3281	9.0820	918.3989	932.8150	908.1984	0.9184	0.9328	0.9082	0.9198	0.0124
	7	289.2058	287.6963	285.9336	0.0273	0.2727	10.1121	10.0593	9.9977	1011.2101	1005.9321	999.7690	1.0112	1.0059	0.9998	1.0056	0.0057
	8	311.8052	325.6068	293.1817	0.0273	0.2727	10.9023	11.3849	10.2511	1090.2292	1138.4867	1025.1120	1.0902	1.1385	1.0251	1.0251	0.0569
60	0	0.0000	0.0000	0.0000	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	160.5748	165.9625	165.7786	0.0273	0.2727	5.6145	5.8029	5.7965	561.4510	580.2891	579.6459	0.5615	0.5803	0.5796	0.5738	0.0107
	2	182.5489	162.0487	187.3351	0.0273	0.2727	6.3828	5.6660	6.5502	638.2836	566.6045	655.0186	0.6383	0.5666	0.6550	0.6200	0.0470
	3	200.5964	209.1120	195.2891	0.0273	0.2727	7.0139	7.3116	6.8283	701.3869	731.1616	682.8299	0.7014	0.7312	0.6828	0.7051	0.0244

	4	221.3879	220.4826	232.4448	0.0273	0.2727	7.7408	7.7092	8.1274	774.0843	770.9192	812.7448	0.7741	0.7709	0.8127	0.7859	0.0233
	5	235.0351	241.9070	234.0925	0.0273	0.2727	8.2180	8.4583	8.1851	821.8021	845.8297	818.5063	0.8218	0.8458	0.8185	0.8287	0.0149
	6	256.4478	256.7551	280.4536	0.0273	0.2727	8.9667	8.9775	9.8061	896.6715	897.7460	980.6080	0.8967	0.8977	0.9806	0.9250	0.0482
	7	261.8538	272.1133	279.7827	0.0273	0.2727	9.1557	9.5145	9.7826	915.5737	951.4461	978.2622	0.9156	0.9514	0.9783	0.9484	0.0315
	8	262.9873	270.7329	257.2243	0.0273	0.2727	9.1954	9.4662	8.9939	919.5372	946.6195	899.3865	0.9195	0.9466	0.8994	0.9218	0.0237
80	0	0.0000	0.0000	0.0000	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	145.5333	158.5813	144.8010	0.0273	0.2727	5.0886	5.5448	5.0630	508.8582	554.4805	506.2976	0.5089	0.5545	0.5063	0.5232	0.0271
	2	165.0591	169.2678	159.0798	0.0273	0.2727	5.7713	5.9185	5.5622	577.1303	591.8462	556.2238	0.5771	0.5918	0.5562	0.5751	0.0179
	3	185.8435	169.0017	195.5885	0.0273	0.2727	6.4980	5.9091	6.8388	649.8030	590.9156	683.8766	0.6498	0.5909	0.6839	0.6415	0.0470
	4	177.5106	181.0087	185.4746	0.0273	0.2727	6.2067	6.3290	6.4851	620.6673	632.8983	648.5132	0.6207	0.6329	0.6485	0.6340	0.0140
	5	206.7465	187.4632	204.3946	0.0273	0.2727	7.2289	6.5547	7.1467	722.8906	655.4664	714.6673	0.7229	0.6555	0.7147	0.6977	0.0368
	6	204.4178	198.4806	209.5549	0.0273	0.2727	7.1475	6.9399	7.3271	714.7483	693.9889	732.7101	0.7147	0.6940	0.7327	0.7138	0.0194
	7	225.1633	207.4511	233.0113	0.0273	0.2727	7.8728	7.2535	8.1472	787.2850	725.3541	814.7255	0.7873	0.7254	0.8147	0.7758	0.0458
	8	224.2384	228.8137	232.4879	0.0273	0.2727	7.8405	8.0005	8.1289	784.0512	800.0489	812.8955	0.7841	0.8000	0.8129	0.7990	0.0145

APPENDIX 5

Ultrasound-assisted Extraction (UAE)

A 10% DUTY CYCLE

Temperature (ºC)	Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)			Mean	Standard Deviation
		A	B	C			A	B	C	A	B	C	A	B	C		
40	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	142.42	142.66	152.95	0.0273	0.2727	4.9799	4.9881	5.3478	497.9886	498.8099	534.7756	0.4980	0.4988	0.5348	0.5105	0.0210
	2	164.58	192.56	188.50	0.0273	0.2727	5.7545	6.7329	6.5909	575.4528	673.2892	659.0957	0.5755	0.6733	0.6591	0.6359	0.0529
	3	188.22	207.89	220.26	0.0273	0.2727	6.5811	7.2688	7.7014	658.1068	726.8830	770.1408	0.6581	0.7269	0.7701	0.7184	0.0565
	4	205.04	227.91	214.40	0.0273	0.2727	7.1691	7.9687	7.4964	716.9145	796.8754	749.6388	0.7169	0.7969	0.7496	0.7545	0.0402
	5	225.75	246.60	227.90	0.0273	0.2727	7.8935	8.6224	7.9684	789.3471	862.2422	796.8407	0.7893	0.8622	0.7968	0.8161	0.0401
	6	234.45	279.34	249.83	0.0273	0.2727	8.1974	9.7672	8.7352	819.7426	976.7173	873.5233	0.8197	0.9767	0.8735	0.8900	0.0798
	7	257.26	292.95	290.16	0.0273	0.2727	8.9951	10.2430	10.1456	899.5119	1024.3044	1014.5613	0.8995	1.0243	1.0146	0.9795	0.0694
	8	261.97	298.82	261.07	0.0273	0.2727	9.1597	10.4483	9.1283	915.9699	1044.8305	912.8326	0.9160	1.0448	0.9128	0.9579	0.0753
50	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	151.20	317.12	299.45	0.0273	0.2727	5.2865	11.0880	10.4702	528.6550	1108.8011	1047.0193	0.5287	1.1088	1.0470	0.8948	0.1076
	2	266.92	285.42	307.62	0.0273	0.2727	9.3328	9.9798	10.7560	933.2793	997.9824	1075.6000	0.9333	0.9980	1.0756	1.0023	0.0713
	3	297.49	370.00	355.75	0.0273	0.2727	10.4016	12.9372	12.4390	1040.1613	1293.7181	1243.8970	1.0402	1.2937	1.2439	1.1926	0.1343
	4	347.65	404.46	326.85	0.0273	0.2727	12.1555	14.1418	11.4283	1215.5537	1414.1811	1142.8306	1.2156	1.4142	1.1428	1.2575	0.1405
	5	369.64	339.12	480.21	0.0273	0.2727	12.9243	11.8574	16.7905	1292.4325	1185.7420	1679.0567	1.2924	1.1857	1.6791	1.3857	0.0754
	6	321.33	422.36	395.21	0.0273	0.2727	11.2355	14.7680	13.8186	1123.5478	1476.7999	1381.8576	1.1235	1.4768	1.3819	1.4293	0.1828
	7	390.22	466.65	413.48	0.0273	0.2727	13.6442	16.3165	14.4573	1364.4187	1631.6566	1445.7338	1.3644	1.6317	1.4457	1.6317	0.1370
	8	458.24	471.84	497.02	0.0273	0.2727	16.022	16.4979	17.3782	1602.2488	1649.7910	1737.8196	1.6022	1.6498	1.7378	1.6633	0.0688

							5										
	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
60	1	169.26	190.98	197.10	0.0273	0.2727	5.9183	6.6778	6.8917	591.8281	667.7802	689.1755	0.5918	0.6678	0.6892	0.6496	0.0512
	2	205.86	234.61	230.53	0.0273	0.2727	7.1981	8.2031	8.0605	719.8071	820.3137	806.0525	0.7198	0.8203	0.8061	0.7821	0.0544
	3	239.22	272.01	260.41	0.0273	0.2727	8.3643	9.5110	9.1053	836.4336	951.0977	910.5265	0.8364	0.9511	0.9105	0.8994	0.0581
	4	300.82	337.64	256.35	0.0273	0.2727	10.5181	11.8055	8.9632	1051.8084	1180.5560	896.3174	1.0518	1.1806	0.8963	1.0429	0.1423
	5	285.92	321.58	330.67	0.0273	0.2727	9.9973	11.2442	11.5620	999.7278	1124.4197	1156.1996	0.9997	1.1244	1.1562	1.0934	0.0827
	6	322.31	362.00	342.51	0.0273	0.2727	11.2696	12.6574	11.9757	1126.9642	1265.7367	1197.5720	1.1270	1.2657	1.1976	1.1968	0.0694
	7	322.39	331.67	381.45	0.0273	0.2727	11.2724	11.5968	13.3374	1127.2454	1159.6847	1333.7431	1.1272	1.1597	1.3337	1.2069	0.1110
	8	394.85	462.79	509.12	0.0273	0.2727	13.8059	16.1815	17.8015	1380.5909	1618.1502	1780.1543	1.3806	1.6182	1.7802	1.3806	0.1146
	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
80	1	148.65	163.66	175.51	0.0273	0.2727	5.1976	5.7222	6.1367	519.7583	572.2226	613.6756	0.5198	0.5722	0.6137	0.5686	0.0471
	2	188.34	189.24	185.97	0.0273	0.2727	6.5853	6.6167	6.5026	658.5291	661.6714	650.2622	0.6585	0.6617	0.6503	0.6568	0.0059
	3	213.22	220.72	215.74	0.0273	0.2727	7.4553	7.7175	7.5435	745.5314	771.7491	754.3495	0.7455	0.7717	0.7543	0.7572	0.0133
	4	223.76	241.49	273.15	0.0273	0.2727	7.8239	8.4437	9.5509	782.3925	844.3738	955.0867	0.7824	0.8444	0.9551	0.8606	0.0875
	5	238.49	265.39	208.85	0.0273	0.2727	8.3387	9.2793	7.3023	833.8669	927.9343	730.2322	0.8339	0.9279	0.7302	0.8307	0.0989
	6	237.33	294.83	280.01	0.0273	0.2727	8.2981	10.3088	9.7907	829.8099	1030.8789	979.0728	0.8298	1.0309	0.9791	0.9466	0.1044
	7	259.58	309.71	291.19	0.0273	0.2727	9.0761	10.8289	10.1814	907.6101	1082.8915	1018.1363	0.9076	1.0829	1.0181	1.0029	0.0886
	8	291.65	339.18	320.25	0.0273	0.2727	10.1976	11.8594	11.1977	1019.7648	1185.9386	1119.7685	1.0198	1.1859	1.1198	1.1085	0.0837

B 20% DUTY CYCLE

Temperature (ºC)	Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)			Mean	Standard Deviation
		A	B	C			A	B	C	A	B	C	A	B	C		
40	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	178.63	211.80	222.00	0.0273	0.2727	6.2459	7.4056	7.7623	624.5933	740.5640	776.2317	0.6246	0.7406	0.7762	0.7138	0.0793
	2	178.94	217.61	226.82	0.0273	0.2727	6.2566	7.6088	7.9307	625.6585	760.8836	793.0678	0.6257	0.7609	0.7931	0.7265	0.0888
	3	230.45	246.88	192.77	0.0273	0.2727	8.0578	8.6323	6.7402	805.7811	863.2296	674.0231	0.8058	0.8632	0.6740	0.7810	0.0970
	4	204.96	237.50	242.28	0.0273	0.2727	7.1663	8.3041	8.4714	716.6267	830.4094	847.1415	0.7166	0.8304	0.8471	0.7981	0.0710
	5	217.72	253.81	261.20	0.0273	0.2727	7.6127	8.8746	9.1329	761.2735	887.4648	913.2902	0.7613	0.8875	0.9133	0.8540	0.0813
	6	226.05	245.43	277.35	0.0273	0.2727	7.9040	8.5814	9.6975	790.3961	858.1392	969.7464	0.7904	0.8581	0.9697	0.8728	0.0906
	7	251.01	283.47	284.53	0.0273	0.2727	8.7766	9.9114	9.9487	877.6590	991.1386	994.8683	0.8777	0.9911	0.9949	0.9546	0.0666
	8	253.56	352.21	307.97	0.0273	0.2727	8.8658	12.3149	10.7681	886.5820	1231.4944	1076.8079	0.8866	1.2315	1.0768	0.9817	0.1728
50	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	268.55	326.98	284.72	0.0273	0.2727	9.3900	11.4327	9.9551	939.0037	1143.2752	995.5114	0.9390	1.1433	0.9955	1.0259	0.1055
	2	295.54	250.98	388.75	0.0273	0.2727	10.3335	8.7754	13.5927	1033.3526	877.5375	1359.2727	1.0334	0.8775	1.3593	1.0901	0.1503
	3	311.65	430.71	324.80	0.0273	0.2727	10.8968	15.0600	11.3566	1089.6860	1505.9978	1135.6632	1.0897	1.5060	1.1357	1.2438	0.1904
	4	394.50	439.11	381.63	0.0273	0.2727	13.7938	15.3536	13.3436	1379.3822	1535.3639	1334.3580	1.3794	1.5354	1.3344	1.4164	0.1055
	5	381.57	471.90	429.94	0.0273	0.2727	13.3416	16.4999	15.0329	1334.1628	1649.9945	1503.2965	1.3342	1.6500	1.5033	1.4958	0.1580
	6	377.84	487.22	487.12	0.0273	0.2727	13.2113	17.0356	17.0321	1321.1320	1703.5571	1703.2080	1.3211	1.7036	1.7032	1.5760	0.0736
	7	478.29	459.52	525.63	0.0273	0.2727	16.7233	16.0671	18.3788	1672.3304	1606.7080	1837.8771	1.6723	1.6067	1.8379	1.7056	0.1191
	8	396.51	428.60	382.95	0.0273	0.2727	13.8639	14.9861	13.3898	1386.3880	1498.6081	1338.9807	1.3864	1.4986	1.3390	1.4080	0.0820
60	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	214.69	223.04	212.71	0.0273	0.2727	7.5065	7.7987	7.4373	750.6499	779.8697	743.7347	0.7506	0.7799	0.7437	0.7581	0.0192
	2	236.66	199.81	240.86	0.0273	0.2727	8.2748	6.9865	8.4216	827.4778	698.6492	842.1591	0.8275	0.6986	0.8422	0.7894	0.0790
	3	206.01	258.83	266.06	0.0273	0.2727	7.2032	9.0499	9.3026	720.3197	904.9888	930.2647	0.7203	0.9050	0.9303	0.8519	0.1146

	4	225.38	265.70	268.87	0.0273	0.2727	7.8805	9.2901	9.4011	788.0500	929.0140	940.1087	0.7881	0.9290	0.9401	0.8857	0.0848
	5	248.64	287.53	343.86	0.0273	0.2727	8.6937	10.0534	12.0231	869.3706	1005.3429	1202.3076	0.8694	1.0053	1.2023	1.0257	0.1674
	6	271.96	299.89	329.98	0.0273	0.2727	9.5092	10.4857	11.5377	950.9246	1048.5705	1153.7722	0.9509	1.0486	1.1538	1.0511	0.1014
	7	273.86	317.07	298.43	0.0273	0.2727	9.5754	11.0863	10.4345	957.5395	1108.6343	1043.4511	0.9575	1.1086	1.0435	1.0365	0.0758
	8	270.01	306.61	311.56	0.0273	0.2727	9.4409	10.7206	10.8937	944.0955	1072.0600	1089.3724	0.9441	1.0721	1.0894	1.0352	0.0794
80	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	91.97	149.08	165.39	0.0273	0.2727	3.2158	5.2127	5.7830	321.5837	521.2726	578.2991	0.3216	0.5213	0.5783	0.4737	0.1348
	2	93.45	158.41	196.55	0.0273	0.2727	3.2676	5.5389	6.8724	326.7558	553.8880	687.2358	0.3268	0.5539	0.6872	0.5226	0.1823
	3	107.04	165.28	209.77	0.0273	0.2727	3.7427	5.7792	7.3345	374.2676	577.9160	733.4500	0.3743	0.5779	0.7335	0.5619	0.1801
	4	229.67	201.94	191.86	0.0273	0.2727	8.0303	7.0609	6.7085	803.0287	706.0906	670.8486	0.8030	0.7061	0.6708	0.7267	0.0684
	5	222.54	257.15	262.71	0.0273	0.2727	7.7810	8.9913	9.1856	778.1054	899.1305	918.5623	0.7781	0.8991	0.9186	0.7781	0.0761
	6	130.74	249.33	263.64	0.0273	0.2727	4.5715	8.7178	9.2183	457.1466	871.7770	921.8307	0.4571	0.8718	0.9218	0.7503	0.0354
	7	218.65	232.35	259.66	0.0273	0.2727	7.6452	8.1240	9.0789	764.5195	812.4041	907.8956	0.7645	0.8124	0.9079	0.8283	0.0730
	8	233.90	249.52	310.60	0.0273	0.2727	8.1782	8.7243	10.8602	817.8182	872.4323	1086.0188	0.8178	0.8724	1.0860	0.9254	0.1417

C 40% DUTY CYCLE

Temperature (ºC)	Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)			Mean	Standard Deviation
		A	B	C			A	B	C	A	B	C	A	B	C		
40	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	252.99	255.22	247.80	0.0273	0.2727	8.8457	8.9238	8.6643	884.5752	892.3777	866.4347	0.8846	0.8924	0.8664	0.8811	0.0133
	2	267.08	259.80	242.34	0.0273	0.2727	9.3383	9.0840	8.4735	933.8303	908.4057	847.3519	0.9338	0.9084	0.8474	0.8965	0.0444
	3	256.08	280.18	290.71	0.0273	0.2727	8.9537	9.7965	10.1646	895.3754	979.6488	1016.4622	0.8954	0.9796	1.0165	0.9638	0.0621
	4	295.30	236.58	306.87	0.0273	0.2727	10.3253	8.2719	10.7296	1032.5322	827.1873	1072.9583	1.0325	0.8272	1.0730	0.9776	0.1318
	5	299.92	293.92	272.48	0.0273	0.2727	10.4866	10.2770	9.5274	1048.6579	1027.7034	952.7379	1.0487	1.0277	0.9527	1.0097	0.0504
	6	321.75	308.57	287.71	0.0273	0.2727	11.2499	10.7893	10.0599	1124.9863	1078.9301	1005.9877	1.1250	1.0789	1.0060	1.0700	0.0600
	7	344.65	301.00	316.88	0.0273	0.2727	12.0506	10.5246	11.0796	1205.0606	1052.4614	1107.9660	1.2051	1.0525	1.1080	1.1218	0.0772
	8	349.49	332.65	375.66	0.0273	0.2727	12.2198	11.6312	13.1349	1221.9829	1163.1245	1313.4868	1.2220	1.1631	1.3135	1.2329	0.0758
50	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	368.40	440.12	408.39	0.0273	0.2727	12.8811	15.3888	14.2793	1288.1111	1538.8796	1427.9273	1.2881	1.5389	1.4279	1.4183	0.1257
	2	267.27	449.08	497.04	0.0273	0.2727	9.3453	15.7019	17.3789	934.5284	1570.1959	1737.8935	0.9345	1.5702	1.7379	1.4142	0.1186
	3	385.99	448.35	408.94	0.0273	0.2727	13.4963	15.6765	14.2985	1349.6321	1567.6489	1429.8517	1.3496	1.5676	1.4299	1.4490	0.1103
	4	415.60	457.82	475.25	0.0273	0.2727	14.5313	16.0077	16.6170	1453.1318	1600.7741	1661.7062	1.4531	1.6008	1.6617	1.5719	0.1072
	5	495.29	507.27	519.40	0.0273	0.2727	17.3177	17.7365	18.1607	1731.7680	1773.6561	1816.0728	1.7318	1.7737	1.8161	1.7738	0.0422
	6	177.88	526.96	305.13	0.0273	0.2727	6.2196	18.4251	10.6690	621.9613	1842.5072	1066.9050	0.6220	1.8425	1.0669	1.8425	0.0772
	7	332.99	519.78	526.68	0.0273	0.2727	11.6429	18.1741	18.4152	1164.2938	1817.4087	1841.5232	1.1643	1.8174	1.8415	1.6077	0.1286
	8	447.31	523.36	402.66	0.0273	0.2727	15.6403	18.2993	14.0791	1564.0282	1829.9277	1407.9165	1.5640	1.8299	1.4079	1.6006	0.2134
60	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	208.47	206.56	238.66	0.0273	0.2727	7.2893	7.2225	8.3449	728.9310	722.2557	834.4872	0.7289	0.7223	0.8345	0.7619	0.0630
	2	228.09	233.80	220.33	0.0273	0.2727	7.9750	8.1748	7.7038	797.5053	817.4833	770.3823	0.7975	0.8175	0.7704	0.7951	0.0236
	3	233.94	234.85	241.01	0.0273	0.2727	8.1796	8.2114	8.4271	817.9593	821.1385	842.7096	0.8180	0.8211	0.8427	0.8273	0.0135

	4	265.40	256.55	252.17	0.0273	0.2727	9.2798	8.9704	8.8170	927.9822	897.0444	881.7059	0.9280	0.8970	0.8817	0.9022	0.0236
	5	306.23	308.08	281.06	0.0273	0.2727	10.7074	10.7719	9.8273	1070.7430	1077.1938	982.7286	1.0707	1.0772	0.9827	0.9827	0.0528
	6	279.80	300.93	278.81	0.0273	0.2727	9.7832	10.5222	9.7485	978.3240	1052.2163	974.8508	0.9783	1.0522	0.9749	0.9766	0.0437
	7	308.96	290.09	286.71	0.0273	0.2727	10.8028	10.1430	10.0249	1080.2817	1014.3038	1002.4916	1.0803	1.0143	1.0025	1.0025	0.0419
	8	287.77	265.92	290.87	0.0273	0.2727	10.0620	9.2980	10.1704	1006.2004	929.7998	1017.0441	1.0062	0.9298	1.0170	0.9843	0.0476
80	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	179.89	189.30	203.04	0.0273	0.2727	6.2899	6.6187	7.0994	628.9890	661.8723	709.9419	0.6290	0.6619	0.7099	0.6669	0.0407
	2	233.86	221.39	208.50	0.0273	0.2727	8.1768	7.7411	7.2902	817.6761	774.1070	729.0222	0.8177	0.7741	0.7290	0.7736	0.0443
	3	210.62	239.28	221.84	0.0273	0.2727	7.3645	8.3663	7.7566	736.4481	836.6283	775.6575	0.7364	0.8366	0.7757	0.7829	0.0505
	4	261.93	211.22	268.38	0.0273	0.2727	9.1583	7.3855	9.3841	915.8292	738.5462	938.4093	0.9158	0.7385	0.9384	0.8643	0.1095
	5	236.08	268.28	227.86	0.0273	0.2727	8.2547	9.3804	7.9672	825.4727	938.0386	796.7191	0.8255	0.9380	0.7967	0.8534	0.0747
	6	263.35	239.24	279.83	0.0273	0.2727	9.2079	8.3650	9.7842	920.7892	836.5013	978.4188	0.9208	0.8365	0.9784	0.9119	0.0714
	7	274.61	301.08	263.64	0.0273	0.2727	9.6016	10.5273	9.2182	960.1590	1052.7329	921.8177	0.9602	1.0527	0.9218	0.9782	0.0673
	8	272.37	291.41	280.46	0.0273	0.2727	9.5233	10.1891	9.8063	952.3300	1018.9064	980.6331	0.9523	1.0189	0.9806	0.9840	0.0334

APPENDIX 6

Enzymatic Extraction (EnE)

Cellulase concentration (g/L)	Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)		
		Mean	Stdev	%stdev			Mean	Stdev	%stdev	Mean	Stdev	%stdev	Mean	Stdev	%stdev
0.025	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	216.40	31.95	14.77	0.0273	0.2727	7.5664	1.1173	14.7661	756.6448	111.7271	14.7661	0.7566	0.1117	14.7661
	2	242.43	29.61	12.21	0.0273	0.2727	8.4767	1.0354	12.2141	847.6748	103.5355	12.2141	0.8477	0.1035	12.2141
	3	269.48	36.00	13.36	0.0273	0.2727	9.4224	1.2586	13.3577	942.2354	125.8610	13.3577	0.9422	0.1259	13.3577
	4	276.61	49.90	18.04	0.0273	0.2727	9.6717	1.7447	18.0393	967.1699	174.4706	18.0393	0.9672	0.1745	18.0393
	5	293.85	57.82	19.68	0.0273	0.2727	10.2744	2.0218	19.6781	1027.4379	202.1799	19.6781	1.0274	0.2022	19.6781
	6	321.64	40.00	12.44	0.0273	0.2727	11.2461	1.3986	12.4365	1124.6072	139.8623	12.4365	1.1246	0.1399	12.4365
	7	341.73	69.55	19.59	0.0273	0.2727	11.9487	2.3411	19.5928	1194.8665	234.1074	19.5928	1.1949	0.2341	19.5928
	8	359.40	25.60	6.99	0.0273	0.2727	12.5665	0.8781	6.9879	1256.6536	87.8134	6.9879	1.2567	0.0878	6.9879
0.050	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	255.97	36.57	14.29	0.0273	0.2727	8.9502	1.2786	14.2861	895.0180	127.8628	14.2861	0.8950	0.1279	14.2861
	2	278.19	51.21	18.41	0.0273	0.2727	9.7271	1.7906	18.4086	972.7077	179.0621	18.4086	0.9727	0.1791	18.4086
	3	273.40	37.21	13.61	0.0273	0.2727	9.5594	1.3010	13.6097	955.9398	130.1001	13.6097	0.9559	0.1301	13.6097
	4	313.27	47.39	15.13	0.0273	0.2727	10.9534	1.6571	15.1284	1095.3373	165.7074	15.1284	1.0953	0.1657	15.1284
	5	328.70	50.96	15.50	0.0273	0.2727	11.4929	1.7819	15.5041	1149.2898	178.1866	15.5041	1.1493	0.1782	15.5041
	6	334.33	8.27	2.47	0.0273	0.2727	11.6899	0.2892	2.4743	1168.9956	28.9245	2.4743	1.1690	0.0289	2.4743
	7	367.42	49.21	13.39	0.0273	0.2727	12.8470	1.7205	13.3925	1284.6991	172.0536	13.3925	1.2847	0.1721	13.3925
	8	367.70	50.32	13.68	0.0273	0.2727	12.8565	1.7594	13.6849	1285.6548	175.9403	13.6849	1.2857	0.1759	13.6849
	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

0.100	1	227.70	30.22	13.27	0.0273	0.2727	7.9615	1.0567	13.2725	796.1489	105.6692	13.2725	0.7961	0.1057	13.2725
	2	267.51	42.04	15.72	0.0273	0.2727	9.3534	1.4701	15.7173	935.3368	147.0096	15.7173	0.9353	0.1470	15.7173
	3	272.73	34.59	12.68	0.0273	0.2727	9.5360	1.2094	12.6821	953.6001	120.9368	12.6821	0.9536	0.1209	12.6821
	4	300.13	62.09	20.69	0.0273	0.2727	10.4942	2.1708	20.6859	1049.4241	217.0831	20.6859	1.0494	0.2171	20.6859
	5	320.21	52.00	16.24	0.0273	0.2727	11.1962	1.8183	16.2403	1119.6247	181.8309	16.2403	1.1196	0.1818	16.2403
	6	334.29	48.34	14.46	0.0273	0.2727	11.6885	1.6902	14.4607	1168.8481	169.0238	14.4607	1.1688	0.1690	14.4607
	7	351.45	49.02	13.95	0.0273	0.2727	12.2885	1.7141	13.9486	1228.8525	171.4080	13.9486	1.2289	0.1714	13.9486
	8	362.96	70.24	19.35	0.0273	0.2727	12.6909	2.4560	19.3523	1269.0933	245.5988	19.3523	1.2691	0.2456	19.3523
0.200	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	213.98	25.87	12.09	0.0273	0.2727	7.4818	0.9046	12.0904	748.1760	90.4571	12.0904	0.7482	0.0905	12.0904
	2	253.06	43.04	17.01	0.0273	0.2727	8.8483	1.5050	17.0088	884.8315	150.4993	17.0088	0.8848	0.1505	17.0088
	3	266.44	37.88	14.22	0.0273	0.2727	9.3162	1.3244	14.2163	931.6215	132.4423	14.2163	0.9316	0.1324	14.2163
	4	276.91	55.13	19.91	0.0273	0.2727	9.6823	1.9277	19.9094	968.2347	192.7696	19.9094	0.9682	0.1928	19.9094
	5	304.14	57.49	18.90	0.0273	0.2727	10.6342	2.0102	18.9033	1063.4240	201.0219	18.9033	1.0634	0.2010	18.9033
	6	309.03	38.81	12.56	0.0273	0.2727	10.8051	1.3568	12.5574	1080.5092	135.6839	12.5574	1.0805	0.1357	12.5574
	7	321.82	42.60	13.24	0.0273	0.2727	11.2524	1.4895	13.2368	1125.2429	148.9463	13.2368	1.1252	0.1489	13.2368
	8	346.20	20.45	5.91	0.0273	0.2727	12.1049	0.7149	5.9057	1210.4943	71.4879	5.9057	1.2105	0.0715	5.9057
0.300	0	0.00	0.00	0.00	0.0273	0.2727	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	1	194.75	31.48	16.16	0.0273	0.2727	6.8094	1.1007	16.1648	680.9404	110.0729	16.1648	0.6809	0.1101	16.1648
	2	200.12	29.85	14.92	0.0273	0.2727	6.9970	1.0439	14.9186	699.7051	104.3863	14.9186	0.6997	0.1044	14.9186
	3	228.97	38.49	16.81	0.0273	0.2727	8.0061	1.3458	16.8094	800.6063	134.5772	16.8094	0.8006	0.1346	16.8094
	4	263.53	46.11	17.50	0.0273	0.2727	9.2145	1.6122	17.4968	921.4496	161.2243	17.4968	0.9214	0.1612	17.4968
	5	261.19	48.27	18.48	0.0273	0.2727	9.1325	1.6879	18.4821	913.2556	168.7884	18.4821	0.9133	0.1688	18.4821
	6	311.35	29.89	9.60	0.0273	0.2727	10.8864	1.0452	9.6006	1088.6392	104.5158	9.6006	1.0886	0.1045	9.6006
	7	311.11	30.00	9.64	0.0273	0.2727	10.8781	1.0490	9.6433	1087.8137	104.9012	9.6433	1.0878	0.1049	9.6433
	8	314.58	49.99	15.89	0.0273	0.2727	10.9992	1.7480	15.8924	1099.9164	174.8034	15.8924	1.0999	0.1748	15.8924

APPENDIX 7

Ultrasound-assisted Enzymatic Extraction (UAEnE)

Time (h)	Area (AU)			Mass (kg)	Volume (l)	Concentration (ppm)			Concentration (mg/kg)			Concentration (mg/g)			Mean	Standard deviation
	A	B	C			A	B	C	A	B	C	A	B	C		
0	377.83	427.78	478.18	0.0273	0.2727	13.2108	14.9575	16.7194	1321.0821	1495.7470	1671.9427	1.3211	1.4957	1.6719	1.4963	0.1754
1	459.61	548.54	591.27	0.0273	0.2727	16.0704	19.1798	20.6739	1607.0458	1917.9790	2067.3953	1.6070	1.9180	2.0674	1.8641	0.2348
2	538.50	606.15	595.23	0.0273	0.2727	18.8288	21.1939	20.8121	1882.8852	2119.3916	2081.2166	1.8829	2.1194	2.0812	2.0278	0.1270
3	563.45	636.64	651.96	0.0273	0.2727	19.7011	22.2600	22.7957	1970.1133	2226.0015	2279.5723	1.9701	2.2260	2.2796	2.1586	0.1654
4	589.61	610.05	692.70	0.0273	0.2727	20.6158	21.3304	24.2201	2061.5775	2133.0411	2422.0153	2.0616	2.1330	2.4220	2.2055	0.1908
5	645.16	775.31	821.95	0.0273	0.2727	22.5581	27.1086	28.7397	2255.8158	2710.8636	2873.9703	2.2558	2.7109	2.8740	2.6135	0.3204
6	604.32	825.32	776.55	0.0273	0.2727	21.1302	28.8575	27.1520	2113.0247	2885.7511	2715.2019	2.1130	2.8858	2.7152	2.8005	0.4060
7	777.20	852.82	882.77	0.0273	0.2727	27.1748	29.8188	30.8660	2717.4787	2981.8852	3086.6008	2.7175	2.9819	3.0866	2.9287	0.1902
8	780.76	688.44	990.55	0.0273	0.2727	27.2994	24.0712	34.6346	2729.9397	2407.1206	3463.4657	2.7299	2.4071	3.4635	2.8668	0.5413

APPENDIX 8
List of Publications

i. Noor Adilah Binti Md Salehan, Ahmad Ziad Bin Sulaiman, Azilah Binti Ajit , (2016), Effect of Temperature and Sonication on the Extractionof Gallic Acid from *Labisia Pumila* (Kacip Fatimah), *ARPN Journal of Engineering and Applied Sciences,* 11(4), 2193-2198.

ii. Noor Adilah Md Salehan, Ahmad Ziad Sulaiman and Azilah Ajit, (2015), Effect of Ultrasound on the Extraction of *Labisia Pumila* (Kacip Fatimah) for the Food Application, *The New Zealand Institute of Food Science & Technology Conference.*

iii. Noor Adilah Binti Md Salehan, Ahmad Ziad Bin Sulaiman, (2015), Effect of Ultrasound on the Extraction of Gallic Acid from *Labisia Pumila* (Kacip Fatimah), *National Postgraduate Conference 2015(NCON-PGR 2015), Universiti Malaysia Pahang, Pahang*, 544-546.

iv. Noor Adilah Binti Md Salehan, Ahmad Ziad Bin Sulaiman, Azilah Binti Ajit, (2014), Effect of Ultrasound and Enzymes on the Extraction of Gallic Acid from *Labisia Pumila* (Kacip Fatimah), *5th International Conference on Biotechnology for the Wellness Industry (ICBWI), J,Teknologi Malaysia, Kuala Lumpur, PS1-*28.

APPENDIX 9
Conferences Presented

i. "The International Conference on Fluids & Chemical Engineering (FluidsChe2015)" on 25th - 27th November 2015.

ii. "The New Zealand Institute of Food Science & Technology Conference (NZIFST)" on 30th June-2nd July 2015.

iii. "National Conference on Postgraduate Research 2015 (NCON-PGR 2015)" on 24th - 25th January 2015.

iv. "5th International Conference on Biotechnology for the Wellness Industry (ICBWI)" on 10th – 11th June 2014.

APPENDIX 10
Attachment of Publication

Publication 1

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EFFECT OF TEMPERATURE AND SONICATION ON THE EXTRACTION OF GALLIC ACID FROM LABISIA PUMILA (KACIP FATIMAH)

Noor Adilah Md Salehan, Ahmad Ziad Sulaiman and Azilah Ajit
Faculty of Chemical Engineering & Natural Resources, Universiti Malaysia Pahang, Lebuhraya Tun Razak, Gambang, Kuantan, Pahang Darul Makmur, Malaysia
Email : ziad@ump.edu.my

ABSTRACT

The increasing demand of herbal based product has created great opportunities for global marketing. *Labisia pumila* contains phenolic compounds and it has been proven to have multiple biological effects, such as high antioxidant properties and anti inflammatory activity. The gallic acid (3,4,5-Trihydroxybenzoic acid) is phenolic compounds that exist in *Labisia Pumila*. Therefore, it is vital to identify the best extraction technique to maximize the performance of the process. Recently, ultrasound-assisted extraction (UAE) widely reported for the extraction of medicinal plants and herbs due to its economic and green technology. The influence of several parameters on the extraction of *Labisia pumila* were investigated : extraction time (1-8 hours), temperature (40,50,60, and 80 °C) , and sonication (40% duty cycle and without sonication) with solvent-to-sample ratio (1:10). The power intensity at 8.66 W/cm² was implemented using ultrasonic processor Q700 (700 watts, 20kHz) provided by QSonica, Newtown, U.S. The study was found that, the gallic acid extract increased with increasing temperature up to 50 °C and 6 hours. Result indicated the extraction of gallic acid may occur to a certain level and then began to declined due to decomposition of the compound.The highest improvement by ultasound-assisted extraction was at 50 °C by 2.26 fold. It can be concluded that, sonication was improved the extraction of bioactive constituents yield without any chemical aid.

Keywords: ultrasound-assisted extraction, gallic acid, intensity.

INTRODUCTION

Labisia pumila,is a genus of small woody and leafy plants belonging to the Myrsinaceae family that can widely found in the tropical forest of South East Asian countries (Chua, Lee, Abdullah, & Sarmidi, 2012). Labisia pumila is a plant with creeping stems and is mainly found in the lowland and hill forests in Southeast Asia, particularly Malaysia, Indonesia, Thailand, Laos, Cambodia, and Vietnam (Farouk, Nawi, & Hassan, 2008) and mostly obtained from the natural tropical forest (Fazwa, Maideen, & Mohamad, 2013). It can be recognized as a small herbaceous under a shrub that roots from the stem with a few leaves pointing upwards with the spike like panicle of small clusters of white or pink flower (Pattiram, Olusegun, Tan, Sarker, & Islam, 2011). There are eight varieties of Labisia pumila (Sunarno, 2005) but only three of the varieties that widely found and studied are Labisia Pumila var. pumila, var. alata and var. Lanceolata (Chua *et al*. 2012). Varieties of Labisia pumila can be differentiated from each other by their petiole and leaf characteristics. Labisia pumila var. Alata has a winged petiole and red veins, while var. pumila has a marginate petiole and ovate leaf blade shape, and var.lanceolata has a long and non-winged petiole. The var. alata is widely used in traditional medicine preparation because it is the most commonly encountered variety in Malaysia. Labisia pumila having high potential in the management of chronic diseases (Nik Hussain & Kadir, 2013).

As mentioned in previous reports, Labisia pumila contains a phenolic compounds (Karimi & Jaafar, 2011) and it has been proven to have multiple biological effects, such as high antioxidant properties (Chua *et al*. 2011) and anti inflammatory activity (Vijayalakshmi & Ravindhran, 2012). The main functions of antioxidants function is to delay the oxidation processes of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals.Arguably this could at least in part be due to the presence of one of the important phytochemical, gallic acid (3,4,5-trihydroxybenzoic acid). The antioxidant activity of benzoic acids has been repoted higher than vitamin C and E agaist reactive oxygen species (Chua *et al*. 2012). The chemical formula of gallic acid is $C_7H_6O_5$ with an exact molecular mass of 170.12 g/mol. Their chemical structures are shown in Figure-1.

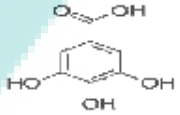

Figure-1. The chemical structures of gallic acid.

Ultrasound-assisted extraction (UAE) has been recognised for potential industrial application in the phyto-pharmaceutical extraction industry for a wide range of herbal extracts. UAE process enhancement for food and allied industries includes herbal, oil, protein and bioactives from plant and animal materials. UAE method able to increase yield of extracted components, increase rate of extraction, achieve reduction in extraction time and higher processing throughput. Vilkhu, Mawson, Simons, and Bates (2008) were reported that, ultrasound can enhanced existing extraction processes and enable new commercial extraction opportunities and processes.

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Living tissues where the desired components are localized in surface glands can be stimulated to release the components by relatively mild ultrasonic stressing (Toma, Vinatoru, Paniwnyk, & Mason, 2001). In tissues where the desired components are located within cells, pre-ultrasound treatment by size reduction to maximise surface area is critical for achieving rapid and complete extraction (Balachandran, Kentish, Mawson, & Ashokkumar, 2006). Devgun, Nanda, and Ansari (2012) reported that ultrasonic-assisted extraction technique enable automation, shortened extraction time and reduce organic solvent consumption. The UAE performance is contributed by the factors of intensity, time, solvent,temperature, pulsation and matrix. Besides that, UAE involve mechanical vibrations which is sound waves with high frequency. Ultrasound can increase in the permeability of the cell wall, mechanical stressing and cavitation effect during the extraction process.

This work focused on examining the effects of ultrasound on the Labisia pumila extraction. Sonication regimens which could influence a process relative to control were identified (model system are using a water). Attempts were made to understand the possible causes of ultrasound-induced enhancement in diverse model of extraction situations. The model processes investigated included: 1) an extraction involving water-based system with the heat; 2) the effect of sonication on the bioactive constituents yield (gallic acid) and to observe the solid-liquid mass transfer limitations.

MATERIALS AND METHODS

Plant Materials

The plant material, Labisia pumila (Kacip Fatimah) were purchased locally from Delima Jelita,Simpang Empat, Alor Setar, Kedah. Prior experiment, the Labisia pumila was grounded and sieved with particle size ~0.3 mm and then stored in the fridge at 4 °C until it used for the experiments.

Conventional Extraction

In this study, sample Labisia pumila were used in powder forms. The particle size was determined in the range of 0.15 to 0.3 mm by sieving using a standard sample sieve and a sieve shaker. Ground Labisia pumila leaves were immersed in the extraction solvent and the mixture was heated on a hotplate with continuous stirring for 8 hours. Four different extraction temperature were applied in this study (40,50,60, and 80 °C) and the sample-to-solvent ratio of each mixture was set at 1: 10 (sample : water) with the volume of infusion was set at 300 ml. The mixture was covered with aluminium foil throughout the extraction to minimize the evaporation in order to maintain the sample-to-water ratio. Then, the sample is centrifuged with speeds 5000 rpm for 10 minutes to separate a heterogeneous mixture of solid and liquid after extraction process. Extracted product was left to cool at room temperature and then was kept at 4 °C prior to analysis for determination of active compounds by using HPLC. The temperature was measured with an external temperature probe. Each experiment was performed in triplicate.

Ultrasound-Assisted Extraction

Ultrasound-assisted extractions of gallic acid from Labisia pumila was conducted by using ultrasonic processor Q700 (700 watts, 20kHz) from QSonica, Newtown, U.S.A with a replaceable flat tip ultrasonic probe (sonotrode) made of titanium alloy that had a tip diameter of 12.7 mm and 127 mm length. The ultrasonic probe was immersed in the extraction medium and the energy is transmitted via the sonotrode directly into the sample. The ultrasound power level was fixed by setting the amplitude of the sonotrode and the cumulative average ultrasound dose by adjusting the duty cycle. The sonication intensity was calculated using the following equation :

$$I = \frac{P}{A}$$

where A (cm^2) was the area of the sonotrode tip. The A value was 1.27 cm^2. The control (conventional) experiments did not use sonication although the sonotrode was installed as in Figure-2. The amplitude was set at position 1 to correspond to a power input P of 11W, and of 8.66 W/cm^2 sonication intensity,I using 40% duty cycles (A duty cycle of 40%, for example,was obtained by sonicating for 4 s followed by a rest period of 6s).

Eight different extraction processes were applied in this study: four conventional processes (at four difference temperature;40,50,60,80 °C) and four innovative ultrasound-assisted extraction process (at four difference temperature;40,50,60,80 °C with 40% duty cycle of sonication).

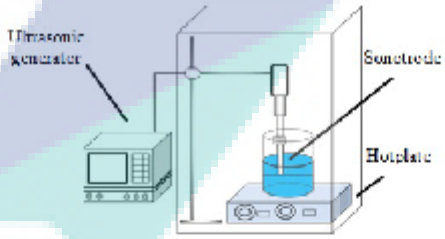

Figure-2. Schematic diagram of ultrasound-assisted extraction.

Analysis of Sample

The measurements of separation and determination of gallic acid were performed using an High Performance Liquid Chromatography (HPLC) system Agilent Series 1100 equipped with diode array detection (DAD) and a column Phenomenex Prodigy 5µ (250 X 4.60 mm). The wavelength for detection of gallic acid was set at 270 nm. Separation was achieved by flow rate of 1

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ml/min with 3.0% Phosphoric acid (90%) / Acetonitrile(10%), in an isocratic programme. The injection volume was 10 µl. Each sample and standard were filtered with nylon syringe filter (pore size of 0.45 µm). For standard preparation,, the mobile phase of phosphoric acid and acetonitrile were prepared, degassed in an ultrasonic bath and will be injected through the chromatographic column.

RESULT AND DISCUSSION

The Chromatographic Separation and Detection of Gallic Acid

Minor modification were made to the chromatographic protocol proposed by Malaysian Standard (2013) for the analysis of gallic acid. The calibration curve for gallic acid was plotted based on the peak areas of chromatograms obtained for various concentrations of standard solutions, prepared from the stock solutions.

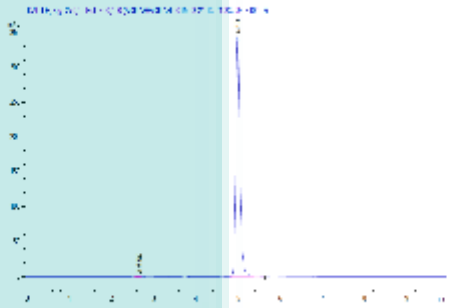

Figure-3. Chromatographic analysis of a gallic acid standard solution(injection volume 10µl).

From the characteristics, HPLC chromatogram of gallic acid (illustrated in Figure-3), it can be observed that a good resolution of the chromatographic peak is obtained. The retention time was 4.9 ± 0.2 min. The calibration curve was obtained by plotting a straight line based on the concentration of gallic acid at 5 differences concentration of standard solution and it shows in Figure-3. An excellent linearity ($R^2 \approx 1$) for gallic acid in the range of 5-80 mg/L concentrations is obtained, with an excellent regression factor.

Figure-4 and Figure-5 shows the yield of gallic acid extracted from Labisia pumila by conventional extraction for 1-4hours and 5-8 hours at different temperature with fixed water-sample ratio. From the data obtained, at 40, 50, 60 and 80oC the extraction yield was gradually increased from 873.365±7.203 to 1026.793±4.538 mg gallic acid/kg dry sample, 640.578±3.293 to 1062.211±2.065 mg gallic acid/kg dry sample, 742.492±7.827 to 1015.490±3.530 mg gallic acid/kg dry sample and 666.350±3.857 to 1086.076±6.910 mg gallic acid/kg dry sample respectively.For the first 5 hours, 40 °C gives the higher yield compared to the other temperature but after 6 hours of extraction the performance for all temperature was approximately stay at the same level. During the extraction process, the molecular diffusion occured once the solvent penetrates the plant matrix. Then solvent will dissolve the solute and brings out the target compound from the matrix. The higher temperature can produce the more energy to enhance the extraction process but, certain antioxidant which extracted at lower temperature can decomposed at higher temperature.Certain antioxidants may mobilize and decomposed at higher temperature(Liyana-Pathirana & Shahidi, 2005).

Table-1. Analytical data of gallic acid obtained from conventional extraction (CE) by high performance liquid chromatography (HPLC) system agilent series 1100.

Temperature (°C)	Time(h)	Gallic Acid Concentration (mg gallic acid/kg dry sample)	
40	1	873.365	±7.203
	2	934.813	±10.511
	3	898.677	±7.986
	4	960.369	±11.021
	5	933.486	±1.458
	6	913.624	±2.979
	7	972.411	±2.469
	8	1026.793	±4.538
50	1	640.578	±3.293
	2	740.658	±2.751
	3	803.178	±7.489
	4	847.354	±2.296
	5	884.251	±3.004
	6	918.871	±3.989
	7	987.265	±2.433
	8	1062.211	±2.065
60	1	742.492	±7.827
	2	797.686	±6.021
	3	833.268	±8.221
	4	846.360	±2.386
	5	867.568	±2.826
	6	930.738	±7.602
	7	993.831	±7.979
	8	1015.490	±3.530
80	1	666.350	±3.857
	2	697.100	±4.637
	3	904.177	±9.332
	4	927.991	±5.367
	5	924.408	±6.495
	6	1014.847	±2.631
	7	946.914	±2.388
	8	1086.076	±6.910

Table-2. Analytical data of gallic acid obtained from ultrasound-assisted extraction (UAE) by high performance liquid chromatography (HPLC) system agilent series 1100.

Temperature (°C)	Time (h)	Gallic Acid Concentration (mg gallic acid/kg dry sample)		Increment (fold)
40	1	898.162	±44.326	1.028
	2	979.240	±132.025	1.048
	3	1045.456	±52.872	1.163
	4	882.704	±13.334	0.919
	5	1008.632	±43.778	1.075
	6	965.584	±62.187	1.013
	7	985.749	±33.473	1.014
	8	1034.240	±41.996	1.007
50	1	1151.684	±110.466	2.266
	2	1529.734	±107.444	2.126
	3	1511.685	±135.889	1.769
	4	1657.058	±118.706	1.672
	5	1777.065	±42.229	2.010
	6	1845.863	±183.146	1.230
	7	1832.798	±17.082	1.631
	8	1803.270	±213.761	1.510
60	1	768.279	±63.073	1.028
	2	796.772	±33.683	0.999
	3	784.337	±50.574	0.941
	4	866.806	±109.656	1.023
	5	894.965	±74.823	0.985
	6	913.564	±71.504	0.982
	7	980.018	±67.406	0.986
	8	986.141	±47.636	0.971
80	1	668.149	±40.787	1.003
	2	775.011	±44.409	1.111
	3	828.776	±13.490	0.916
	4	903.888	±23.615	0.974
	5	1011.539	±50.522	1.094
	6	1071.917	±60.112	1.016
	7	1123.873	±77.379	1.187
	8	1235.110	±75.907	1.065

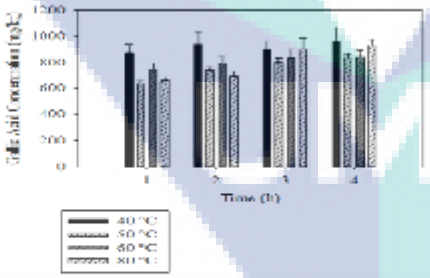

Figure-4. Extraction yield of gallic acid from conventional extraction method at different temperature with 10:1 water to sample ratio for 1-4 hours.

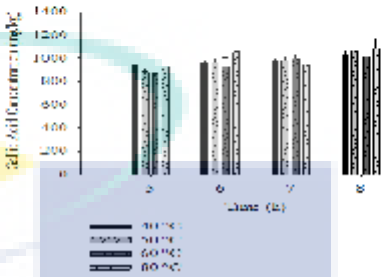

Figure-5. Extraction yield of gallic acid from from conventional extraction method at different temperature with 10:1 water to sample ratio for 5-8 hours.

Effect of sonication on the extraction yield of Labisia pumila at 40, 50, 60 and 80 °C with fixed duty cycle 40% showed in Figure-6 and Figure-7. In general, ultrasound-assited extraction was improved and accelerated the extraction processes. From the data obtained, the highest gallic acid yield is observed at 50 °C after 6 hours extraction with 1845.863±183.146 mg gallic acid/kg dry sample. The extraction process was improved due to sonochemistry effect on the molecular and microstructure of the cell wall. Comparing with the highest yield from conventional extraction, this method just need less than 1 hour to have the same amount of yield at 80 °C after 8 hours extraction process. It was proved that the ultrasound-assisted extraction has accelerated the extraction process and shorten the process time. Acoustic power produced by sonication provide the mechanical effects which results to distruption of cell wall and gives greater penetration of solvent into the cellular materials which leads to facilitate the gallic acid extraction from Labisia pumila.

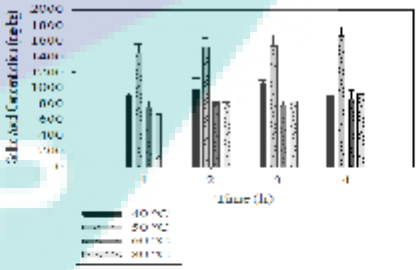

Figure-6. Extraction yield of gallic acid from ultrasound-assisted extraction method at different temperature with 10:1 water to sample ratio for 1-4 hours.

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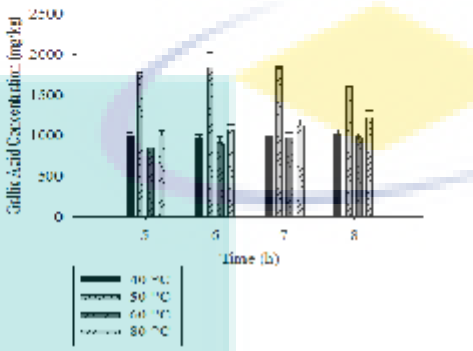


Figure-7. Extraction yield of gallic acid from from ultrasound-assisted extraction method at different temperature with 10:1 water to sample ratio for 5-8 hours.

Figure-8 indicated the highest improvement of gallic acid yield extracted from Labisia pumila by conventional and ultrasound-assisted extraction for each temperature. The highest improvement was at 50 °C after 1 hour extraction process by 2.26 fold. Figure-9 showed the comparison of extraction yield for 8 hours extraction at 50 °C . The target compund of gallic acid was a intracellular compund and it was not freely available. Hence, the improvement by the ultrasound-assisted extraction due to sonication was not only facilitate the process but the energy forms was loosen the matrix and the chemical bonds in the cell wall.

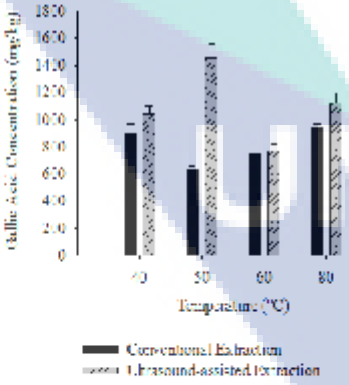


Figure-8. Comparison performance of conventional and ultrasound assisted extraction at 40,50,60 and 80 °C by 8.66 W/cm² ultrasound intensity.

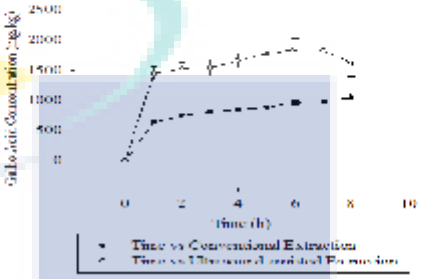
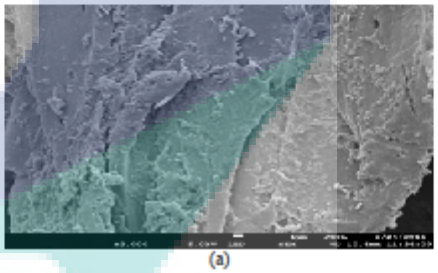
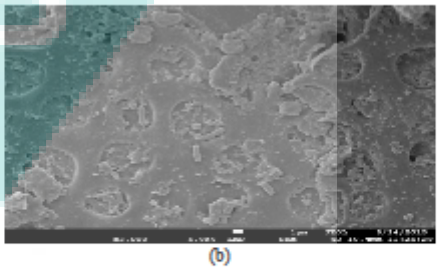


Figure-9. Comparison performance of conventional and ultrasound assisted extraction at 50 °C by 8.66 W/cm² ultrasound intensity for 8 hours.

The extracted Labisia pumila was examined by FESEM to investigate the effect of the different extraction methods on the physical structure of the fine powder. Based on Figure-10, there is no severe fracture was observed during the conventional extraction except few slight ruptures on the surface of the sample. In case of ultrasound –assisted extraction, the swelling and softening process of the cell wall was observed. The effect of acoustic power produced by sonication clearly seen on the Figure-10(a). This, helping in permeation processes of the desired compound out of the matrix.



(a)



(b)

Figure-10. The structures of extracted L.pumila a) FESEM images by conventional extraction with 3,000x magnification; b) FESEM images by ultrasound-assited extraction with 3,000x magnification.

CONCLUSIONS

This research was carried out to determine the performance of ultrasound-assisted extraction method in extraction of gallic acid from Labisia pumila. The maximum gallic acid yield extracted was at temperature 50 °C with sonication intensity 8.66 W/cm² and 40% duty cycle by 2.26 fold increment.The efficiency of the ultrasound-assisted extraction procedure exceed the conventional extraction by improving the yield and shorten the extraction time.

ACKNOWLEDGEMENTS

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Publication 2

ABSTRACTS FOR POSTER PRESENTATION

PS1-27

Effect Of Ultrasound And Enzyme On The Extraction Of *Tongkat Ali*

He Yuhai[1], Ahmad Ziad Bin Sulaiman[1]

Faculty of Chemical & Natural Resources Engineering,Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Kuantan, Pahang.

Abstract

Tongkat Ali, or Eurycoma Longifolia, is a traditional Malay and Orang Asli herb used as aphrodisiac, general tonic, anti-Malaria, and anti-Pyretic. It has been recognized as a cashcrop by Malaysia due to its high value for the pharmaceutical use. In tongkat ali, eurycomanone, a quassinoid is usually chosen as a marker phytochemical as it is the most abundant phytochemical. In this research, ultrasound and enzyme were used to enhance the extraction of Eurycomanone from Tongkat Ali. Ultrasonic assisted extraction (USE) enhances extraction by facilitating the swelling and hydration of the plant material, enlarging the plant pores, breaking the plant cell, reducing the plant particle size and creating cavitation bubbles that enhance mass transfer in both the washing and diffusion phase of extraction. Enzyme hydrolyses the cell wall of the plant, loosening the structure of the cell wall, releasing more phytochemicals from the plant cell, enhancing the productivity of the extraction. Possible effects of ultrasound on the activity of the enzyme during the hydrolysis of the cell wall is under the investigation by this research. The extracts was analysed by high performance liquid chromatography for the yields of Eurycomanone. In this whole process, the conventional water extraction was used as a control of comparing the performance of the ultrasound and enzyme assisted extraction.

Keywords: *Eurycoma Longifolia*, extraction, eurycomanone, ultrasound, enzymes.

PS1-28

Effect of Ultrasound and Enzymes on the Extraction of Gallic Acid from *Labisia Pumila* (Kacip Fatimah)

Noor Adilah Binti Md Salehan, Ahmad Ziad Bin Sulaiman, Azilah Binti Ajit

Faculty of Chemical & Natural Resources Engineering, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Kuantan, Pahang.

Abstract

Ultrasound and enzymes are unified to extract gallic acid from *Labisia pumila* (Kacip Fatimah). Gallic acid has anti-fungal and anti viral properties and acts as an antioxidant, which helps to protect cells against oxidative damage. Gallic acid also shows cytotoxicity against cancer cells, without harming healthy cells. Besides the effect of ultrasound and the activity of *Trichorderma reesei* cellulase on the extraction productivity, solid liquid mass transfer limitations were investigated. The effects of extraction time, extraction temperature, solvent-to-solid ratio, and sonication power on the extraction of gallic acid were parameters considered into account for the research. The unified ultrasound and enzymatic extraction were carried out at low intensity sonication (2.4-11.8 W/cm^2) using a sonicator probe in a designed reactor with optimum conditions for the cellulase reaction (pH 4.8, 40-50 °C). Formation of cavitation bubbles by applying ultrasound enhances both extraction process and enzyme activity through the solid-liquid mass transfer by enlarging the plant pores, breaking the plant cell and reducing the plant particle size. The cellulolytic activity increased by some changes in the spatial structure of enzyme molecules, which supported the formation of enzyme substrate complex and improved the absorption of cellulase on insoluble cellulose. Enzyme hydrolysis enhancing the productivity of the extraction by loosening the network of the cell wall, then releasing more intracellular compound from the plant cell. The extraction of gallic acid was analyzed by using high performance liquid chromatography. Meanwhile the conventional water extraction was used as a control by comparing the performance of the ultrasound and enzyme assisted extraction.

Keywords: extraction, gallic acid, ultrasound,cavitation, cellulose, hydrolysis.

Publication 3

NZIFST Conference 2015 Palmerston North

Effect of Ultrasound on the Extraction of Gallic Acid from Labisia **pumila** (Kacip Fatimah) for the food applications

Noor Adilah Md Salehan, Ahmad Ziad Sulaiman, Azilah Ajit
Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Lebuhraya Tun Razak,26300 Kuantan, Pahang, Malaysia.
ziad@ump.edu.my

The changes in consumer habits of eating and related health problems have led to a growing demand for herbal based products. Recently, ultrasound-assisted extraction (UAE) has been increasingly reported for extraction from, medicinal plants and herbs due to its economic and green technology. The advantages of using ultrasound for extraction include: enhanced mixing, facilitated energy and mass transfer, and also faster start-up processes, with all of these factors lead to increased production. Ultrasound can also enhance existing extraction processes and enable new commercial extraction opportunities and processes. UAE involves mechanical vibrations derived from sound waves with high power and intensity. Ultrasound can increase in the permeability of the cell wall through mechanical stressing and cavitation effect during the extraction process. This study was carried out to determine the performance of UAE in the extraction of gallic acid from Labisia Pumila (Kacip Fatimah). Labisia pumila is a small herbaceous shrub that roots from the stem with a few leaves pointing upwards white or pink flower. Labisia pumila contains phenolic compounds which have been proven to have multiple biological effects, such as high antioxidant properties and anti-inflammatory activity. The main function of antioxidants is to delay the oxidation processes of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals. One important phenolic compound with such antioxidant properties is gallic acid (3,4,5-Trihydroxybenzoic acid). The effect of processing parameters, time and sonication regiments (power intensity and duty cycle) have been studied along with the extraction performance of gallic acid from Labisia pumila. Extraction was conducted by using an ultrasonic processor Q700 (700 watts, 20kHz) provided by QSonica, Newtown, U.S.A with a replaceable flat tip ultrasonic probe (sonotrode) made of titanium alloy that had a tip diameter of 12.7 mm and 127 mm length. The sonication regiments (power intensity and duty cycles) were varied to find the maximum extractable gallic acid concentration. The sonication intensity was calculated using equation I=P/A where A (cm^2) was the area of the sonotrode tip. The A value was 1.27 cm^2. The power intensity was varies (0 - 73.23 $W/cm^{2)}$ for the power level tested and sonication duty was set at 10% , 20% and 100%. A 20% of duty cycle at low power intensity (8.66 W/cm^2) was found to accelerate the extraction process and gave the highest extraction of gallic acid. For the 8 hours extraction, with 20% of duty cycle, the extraction performance was increased by 2-fold. In addition, the processing time was shortened from 8 to 3 hours using a 20% duty cycle to achieve the maximum productivity. This extraction has been successfully done without any chemical aid. In conclusion, ultrasound assisted extraction is an efficient method to improve the extraction efficiency due to its sonochemical effect on the molecular and microstructure of the cell walls of plant.

Publication 4

National Conference for Postgraduates Research 2015

Effect of Ultrasound on the Extraction of Gallic Acid from Labisia Pumila (Kacip Fatimah)

Noor Adilah Md Salehan[a], Ahmad Ziad Sulaiman[b]

[a,b] Faculty of Chemical Engineering & Natural Resources, Universiti Malaysia Pahang, Lebuharaya Tun Razak. 26300 Gambang, Kuantan, Pahang, Malaysia

*Corresponding Author's E-mail : ziad@ump.edu.my

Keywords: Labisa pumila, Gallic acid, Ultrasound-Assisted Extraction (UAE) , power intensity, duty cycle

ABSTRACT

Labisia pumila, is a genus of small woody and leafy plants belonging to the Myrsinaceae family that can widely found in the tropical forest of South East Asian countries (Lee Suan Chua et al., 2012). It has a creeping stems and is mainly found in the lowland and hill forests in Southeast Asia, particularly Malaysia, Indonesia, Thailand, Laos, Cambodia, and Vietnam (Farouk et. al., 2008) and mostly obtained from the natural tropical forest (Md Ariff et. al., 2013). Labisia pumila be recognized as a small herbaceous under a shrub that roots from the stem with a few leaves pointing upwards with the spike like panicle of small clusters of white or pink flower (Pattiram, P. D et al 2011). Labisia pumila contains a phenolic compounds (Siti Nadiah Mohd Abdah,2013) and it has been proven to have multiple biological effects, such as high antioxidant properties (Lee Suan Chua et al., 2012) and anti inflammatory activity (R.Vijayalakshmi and R.Ravindhran, 2012). The main functions of antioxidants function is to delay the oxidation processes of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals. The important phenolic compounds that helps for the process is known as Gallic acid(3,4,5-Trihydroxybenzoic acid). The antioxidant activity of benzoic acids have been reported higher than vitamin C and E agaist reactive oxygen species(Lee Suan Chua et al., 2012).The chemical formula of gallic acid is $C_7H_6O_5$ with an exact molecular mass of 170.12 g/mol. Their chemical structures are shown in Figure 1.The increasing demand of herbal based product has created great opportunities for global marketing. Therefore, it is vital to identify a best extraction technique to maximize the performance of the process. Recently, ultrasound-assisted extraction (UAE) widely reported for the extraction of medicinal plants and herbs due to its economic and green technology. Kamaljit Vilkhu et. al., (2006) was reported that, ultrasound can enhance existing extraction processes and enable new commercial extraction opportunities and processes. UAE involve mechanical vibrations which is sound waves with high power and intensity. Ultrasound can increase in the permeability of the cell wall, mechanical stressing and cavitation effect during the extraction process (Manish Devgun *et. al*, 2012). This study was carried out to determine the performance of UAE in the extraction of gallic acid from *Labisia Pumila* (Kacip Fatimah). The extraction was conducted by using ultrasonic processor Q700 (700 watts, 20kHz) provided by QSonica, Newtown, U.S.A with a replaceable flat tip ultrasonic probe (sonotrode) made of titanium alloy that had a tip diameter of 12.7 mm and 127 mm length. The sonication regiments (power intensity and duty cycles) were varied to find the maximum gallic acid concentration. The ultrasound power level was varied by adjusting the power setting (knobe) of the sonotrode and duty cycle. The setup of the experiments have been shown in Figure 2. The sonication intensity was calculated using Equation 1:

$$I=P/A \quad (1)$$

where A (cm^2) was the area of the sonotrode tip. The A value was 1.27 cm^2. The amplitude was set at position 1,5,10,20,40 and 80 and for the power level tested, sonication duty was set at 5,10,20,40,80 and 100% (A duty cycle of 5%, for example,was obtained by sonicating for 1 s followed by a rest period of 19s). Throughout the experiment, the sample-to-water ratio and temperature was fixed 1:10 and 40°C, respectively for an hour. The measurements of separation and determination of gallic acid were performed using an High Performance Liquid Chromatography (HPLC) system Agilent Series 1100 equipped with Diode Array Detection (DAD) and a column Phenomenex Prodigy 5µ (250 X 4.60 mm). The wavelength for detection of gallic acid was set at 270 nm. Separation was achieved by flow rate of 1 ml/min with 3.0% Phosphoric acid (90%) / Acetonitrile(10%), in an isocratic programme. The injection volume was 10 µl. Each sample and standard were filtered with nylon syringe filter (pore size of 0.45 µm). As a result, at the 40% of duty cycle and low power intensity (8.66 W/cm^2) showed that the maximum gallic acid extraction with 1.2 and 1.3 fold increment respectively. This extraction has been successfully done without any chemical aid.

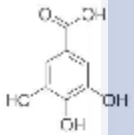

Figure 1. The chemical structures of gallic acid

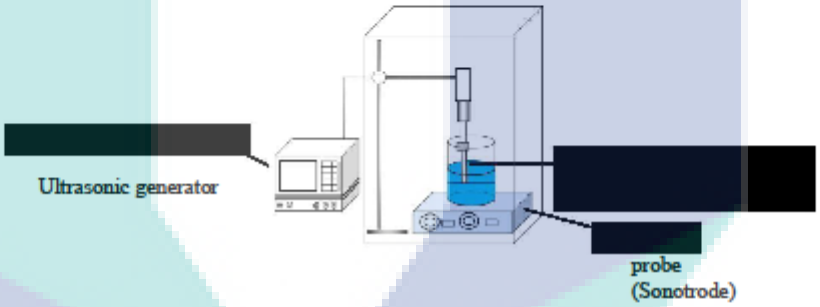

Figure 2. Schematic diagram of ultrasound-assisted extraction

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