MICROWAVE – ASSISTED EXTRACTION (MAE) OF RHIZOME *KAEMPFERIA PARVIFLORA* CRUDE AND ITS BIOLOGICAL ACTIVITY

by

MOHAMAD ANWAR BIN MOHAMMAD GIAL

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LIST OF SYMBOLS

ε'	Dielectric constant
ε''	Dielectric loss
δ	Dissipation factor
A_O	Absorbance value of the control reaction
A_S	Absorbance value of the sample
N	Number of experiments
n	Number of factors or variables
nc	Number of center points
$V_{diluted}$	Volume of dilution
V_{stock}	Volume of stock solution
W_f	Weight of dried extract after freeze drying process
W_O	Weight of powdered rhizome used in the extraction

LIST OF ABBREVIATIONS

AAE	Ascorbic acid equivalence
ANOVA	Analysis of Variance
CCD	Central composite design
CCC	Central composite circumscribed
CCF	Central composite face-centered
CCI	Central composite inscribed
DW	Dry weight
DOE	Design of experiment
DPPH	2, 2′-diphenyl-1-picryl-hydrazyl
GA	Gallic acid
GAE	Gallic acid equivalence
K. Parviflora	Kaempferia Parviflora
LOF	Lack of fit
MAE	Microwave assisted extraction
RSM	Response surface methodology
TPC	Total phenolic content
USM	Universiti Sains Malaysia

PENGEKSTRAKAN TERBANTU GELOMBANG MIRO RIMPANG MENTAH *KAEMPFERIA PARVIFLORA* DAN AKTIVITI BIOLOGINYA

ABSTRAK

Pengekstrakan adalah proses untuk memisahkan produk semulajadi yang dikehendaki dari bahan mentah. Ia telah digunakan dalam pelbagai industri seperti industri makanan dan farmaseutikal untuk mencapai tahap nutrien tertentu yang telah ditetapkan dalam pengeluaran produk. Walau bagaimanapun, teknik pengekstrakan tradisional memerlukan isipadu pelarut yang besar dan memerlukan masa yang lebih panjang untuk pengekstrakan. Oleh itu, pengekstrakan maju merupakan pengekstrakan dengan bantuan gelombang mikro telah dijalankan. Kajian penyelidikan ini mengetengahkan pengekstrakan mesra alam di mana air digunakan sebagai pelarut. Pengoptimuman pengekstrakan bantuan gelombang mikro telah dioptimumkan melalui Kaedah Sambutan Permukaan (RSM) untuk meningkatkan pengekstrakan sari mentah *K. Parviflora*. Pengoptimuman telah selesai dijalankan untuk mendapatkan kuasa optimum gelombang mikro, masa pengekstrakan di dalam gelombang mikro dan nisbah pelarut kepada bahan dengan tindak balas yang tinggi. Reka bentuk Komposit Pusat (CCD) telah dipilih untuk model RSM dalam menilai urutan polinomial pertama dan kedua. Analisis variasi telah digunakan untuk menilai keseragaman model dan keadaan optimum. Dengan mempertimbangkan kandungan hasil pengekstrakan maksimum, jumlah kandungan fenolik dan aktiviti anti-oksida, keadaan optimum untuk semua tindak balas adalah pada kuasa gelombang mikro 360 W, masa gelombang mikro 2 minit dan nisbah pelarut ke bahan 10:1. Dalam keadaan optimum, bahan mentah *K. Parviflora* yang telah diekstrak memperoleh hasil ekstrak sebanyak 16.72%, jumlah kandungan fenol 18.24 μg/ml dan aktiviti anti-oksida 142.681 μg/ml. Kajian ini telah mendedahkan bahawa Kaedah Sambutan Permukaan (RSM) adalah kaedah statistik yang berkesan untuk menyediakan model empirikal yang sesuai kepada pemboleh ubah bebas dan meramalkan keadaan optimum yang mempengaruhi ekstrak mentah *K. Parviflora.* Pengekstrakan dibantu gelombang mikro adalah teknik mesra alam untuk pengekstrakan sebatian bio-aktif dan prosedur alternatif yang menarik dalam industri makan dan tradisional herba.

MICROWAVE-ASSISTED EXTRACTION (MAE) OF RHIZOME *K. PARVIFLORA* CRUDE AND ITS BIOLOGICAL ACTIVITY

ABSTRACT

Extraction is a process to separate the desired natural products from the raw materials. It has been used in various industry such as food and pharmaceutical industries to achieve certain level of nutrients in the products. However, traditional extraction method required a large volume of solvents and longer extraction time. Hence, advanced extraction which is microwave-assisted extraction was carried out. This research study focusses on green extraction technique where the solvent used is water. Microwave-assisted extraction (MAE) was optimized by Response Surface Methodology (RSM) to enhance the extraction of rhizome *K. Parviflora* crude. The optimization was done to get the optimum microwave power, microwave extraction time and solvent to feed ratio with maximum response. Central Composite Design (CCD) was selected as a model for RSM to evaluate the first and second-order polynomial model. The analysis of variance was used to evaluate the model fitness and optimal condition. Considering the maximum content of extracted yield, total phenolic content and antioxidant activity, the optimal conditions for all investigated response were obtained at microwave power of 360 W, microwave time of 2 min and solvent feed ratio of (10:1). Under the optimal condition, obtained *K. Parviflora* crude extract contained 16.72% yield of crude extract, 18.24 μg /ml of total phenolic content and 142.681 μg/ml of antioxidant activity. The study revealed that the response surface methodology (RSM) is an efficient statistical method for preparing appropriate empirical model related to the independent variables and predicting the optimum conditions influencing *K. Parviflora* crude extract. Microwave-assisted extraction is an environmental-friendly technique for extractions of bioactive compounds and an attractive alternative procedure in industry food and traditional herb.

CHAPTER 1

INTRODUCTION

This chapter introduces the overview of this research and significance of microwave assisted extraction for *K. Parviflora* crude. In general, this chapter summarizes the research background of extraction of *K. Parviflora* by using microwave, the problem statement and the objectives of this final year project.

1.1 Background Study

Kaempferia Parviflora wall. ex Baker is herbaceous plant in the family of Zingiberaceae that commonly grown in the tropical Asia especially in the upper North-eastern regions of Thailand (Pitakpawasutthi et al., 2018). This perennial herb is called as rhizome black ginger due to its colour inside the rhizome which is dark purple to black rhizome with brown colour outside (Yee et al., 2019). Even though there were inflated request for rhizomes of *K. Parviflora*, there is deficiency of its planting material in Malaysia which causes sluggish natural regeneration of *K. Parviflora* through rhizome and long dormancy period (Labrooy, 2013). *K. Parviflora* rhizome contain abundant amount of phenolic and flavonoid compound.

Figure 1.1 Rhizome of *Kaempferia Parviflora* or black ginger

K. Parviflora rhizome exhibits biological activities such as anti-gastric ulcer, anti-mycobacterial, anti-inflammatory, anti-allergenic and anti-mutagenic activities (Lee et al., 2018; Hanmontree et al, 2020; Pitakpawasutthi et al., 2018). It has been used as an ingredient in health tonic in Thailand which also locally known as *Kra-chai-dam* (Lee et al., 2018). It also produced food supplement or traditional medicine in form of capsules and tablets. However, using *K. Parviflora* in food products is limited which included the production of wine, yogurt and fermented juice. The solid-liquid extraction being used in production of food supplement where it have been approved by the Thai FDA (Thai Food and Drug Administration) which recommends not more than 200 mg/day of *K. Parviflora* (Hanmontree et al, 2020).

Extraction is process of separation from the raw material to obtain their phytoconstituents compound in the product by using different type of solvents. The efficiency of extraction will depend on several parameter such as solvent extraction, temperature, time, solvent solid ratio and size of raw materials. Method of extraction can be classified into two mode which conventional and advanced extraction.

The conventional extraction methods including Soxhlet, maceration and percolation which normally used as a model to make an efficiency comparison to the advanced extraction (Osorio-Tobón, 2020). This method inherent several disadvantages such as high temperature and longer extraction duration which can cause degradation of phenolic compounds(Osorio-Tobón, 2020). Thus, microwave assisted extraction as an advanced method that have been used in extraction of natural products that rich in phenolic compound and only requires lesser organic solvent consumption, reduction time of extraction and higher selectivity (Zhang et al., 2018).

Microwave-assisted extraction has been acknowledged as method for the extraction of an organic compound from *K. Parviflora* rhizome. The microwave is an

electromagnetic wave with non-ionizing that generate frequency in range of 300.MHz to 300 GHz. It is located between the X-ray and infrared rays in the electromagnetic spectrum. The energy transfer in microwave heating process occur by two process which dipole rotation and ionic conduction. The energy produced from the microwave will directly delivered to material through molecular interactions by transformations of electromagnetic energy into thermal energy (Veggi et al., 2013).

1.2 Problem Statement

Extraction process has been improvised with more efficient and environmental-friendly technique in herbal research, pharmaceuticals and food industry to obtain high quality of phyto-constituents in the plants. However, conventional method for the extraction of active compounds are thermally unsafe which lead to the degradation of phyto-constituents in plant and the extraction step is limited for the analysis of various constituents in plant material. The conventional extraction method inherent several disadvantages such as requires high volume of solvent to increase the extraction efficiency and requires more energy. The purification process and condensation of the solution requires more time to ensure higher yield of phyto-constituents in the obtained crude extracts. In this study of microwave assisted extraction, the process of extraction is different from conventional extraction methods (solid-liquid or simple extraction). For example, soxhlet technique requires the solvent and sample to be heated continuously until it reach the boiling point of solvent by using more extraction time, subsequently causes fewer phenolic compound were collected (Osorio-Tobón, 2020). On the other hand, microwave assisted extraction method provide alternative ways for better extraction efficiency, reduce extraction time, preventing pollution to the environment, less solvent consumption and less energy requirement (Veggi et al., 2013).

Additionally, choosing a suitable solvent need to be considered wisely in order to optimize MAE process (in term of extract yield) and minimize the pollution caused by excessive solvent. The conventional extraction commonly used solvent such as hexane that can be defined as toxic and hazardous solvent which can cause negative impact on health effect if inhaled or digested. In this study, water is being used in the extraction step as a green solvent because it is considered as safe to human and surrounding in fact it is non-flammable, inexpensive and prevent pollution. Therefore, water is the choice of solvent as it imposes lesser effect towards environmental sustainability.

1.3 Objective

The objective of this study are:

i. To extract crude *Kaempferia Parviflora* using microwave assisted extraction method by using water as a solvent.
ii. To optimize the microwave assisted extraction of crude *Kaempferia Parviflora* by manipulating the parameter which were extraction time, microwave power and solid ratio using Design Expert software.
iii. To determine the extraction yield, phenolic content and antioxidant properties of *Kaempferia Parviflora* crude extract.

1.4 Organization of Thesis

This thesis consists of five main chapters which construct the whole thesis.

Chapter one presents the overview and background of the research. A brief introduction to the research, problem statement, objectives and scope of study of the research

Chapter two presents the literature reviews of previous researches on the extraction of *K. Parviflora* crude via microwave assisted extraction and the optimization of extraction via response methodology surface. Sustainability of the research is discussed as well.

Chapter three presents list of materials and chemicals used throughout the research. The procedure of research is constructed in details which consists of Design of Experiments, pre-extraction, microwave-assisted extraction, post-extraction and data collection.

Chapter four presents experimental results and discussion that separated into two main parts which are single-factor analysis and design of experiment.

Chapter five presents the conclusion that reflects the goal of all objectives in this study and recommendations for future research.

CHAPTER 2

LITERATURE REVIEW

2.1 Kaempferia Parviflora

The genus *Kaempferia* L. (Zingiberaceae) is medicinal plant that grow in the tropical Asia region with rich source of biologically active compounds. It is a medium-sized genus with approximately 50 species widely distributed throughout particularly in Southeast Asia which originated from India, South China to Malaysia. The species of Zingiberaceae in Southeast Asia region used it as a spices, traditional medicines, flavouring agent and source of certain dyes (Yee et al., 2019). In Malaysia, *Kaempferia L*. have been used as a treatment of several diseases such as stomach ailments, vomiting, cough, bruises, sore throat, asthma, fever and muscular pains (Elshamy et al., 2019).

This rhizome of black ginger is also commonly grown in the tropical Asia especially in the upper North-eastern regions of Thailand (Pitakpawasutthi et al., 2018). In Thailand, it is locally known as Krachaidum or Black Ginger or Thai Ginseng that used to drink from the sliced black ginger with a tea boiled as well as soaked into the alcohol with black ginger. This perennial herb is called as rhizome black ginger due to its colour inside the rhizome which is dark purple to black rhizome with brown colour outside (Yee et al., 2019). Additionally, the rhizome of *K. Parviflora* has been produced as tonic drink and aphrodisiac that commercially available in the market for wine preparation. As dietary supplements, it has been made into various productions such as medicinal liquor or liquor plus honey, pills (powdered rhizome with honey), capsules and tablets (Toda et al., 2016).

The flavonoids are the major chemical constituents in the *K. Parviflora* extract which have at least 11 methoxyflavones detected by using gas chromatography technique (Rahman et al., 2018). Its rhizomes have been used as folk medicine with

beneficial therapeutic effects for the treatment of a various illness such as treatment of gout, abscesses and colic disorder due to their high contents of biologically active phenolic and methoxyflavone compounds (Yee et al., 2019). The methoxyflavone derivatives also used for treatment of gout, aphthous ulcers, abscesses, allergy and gastrointestinal disorders and aphrodisiac. Additionally, *K. Parviflora* also contains phenolic glycoside and various of flavonoids such 5-hydroxy-7-methoxyflavone, 5,7-dimethoxyflavone, and 3,5,7-trimethoxyflavone (Rahman et al., 2018).

Table 2.1 shows the comparison of total phenolic content and total flavonoids content for different sources of *K. Parviflora* rhizome. The highest total phenolic content and total flavonoid content obtained from roots with the highest production rate or productivity than those from the intact rhizome of a 10 month old plant.

Table 2.1 Comparison of total phenolic content and total flavonoids content for different source of *K. Parviflora* rhizome (Kitwetchar et al., 2020)

Source	**Total Phenolic**		**Total flavonoids**	
	Concentration (µg GAE g^{-1} DW)	**Productivity (µg GAE d^{-1})**	**Concentration (µg GAE g^{-1} DW)**	**Productivity (µg GAE d^{-1})**
Cell culture from roots	343.378 ± 2.04	42.97	292.67 ± 3.02	36.58
Cell culture form shoots	301.78 ± 1.89	37.72	269.33 ± 2.64	33.67
Cell culture form rhizome	142.00 ± 1.60	17.75	150.44 ± 1.86	18.81
Rhizome from 10 month old plant	1,207.00 ± 5.68	4.02	1,077.00 ± 4.45	3.59

Table 2.2 shows the total phenolic content and total flavonoid content from *K. Parviflora* crude extract using solvent extraction method. The highest total phenolic content and total flavonoid content obtained from 8 months old rhizome, while the optimum extraction time in solvent extraction using water as a solvent was 120 minute and at temperature of 90°C. The phenolic and flavonoid content can be increased high temperature being applied, hence it reflected better efficiency of their solubility, extraction rate, diffusion rate, reduced surface tension and solvent viscosity (Rahman et al., 2018).

Table 2.2 Total phenolic content and total flavonoid content from *K. Parviflora* crude extract using solvent extraction method (Rahman et al., 2018)

Phytoconstituent	**Result**
Phenolic Contents	74.3 mg GAE / g dry weight
Flavonoid Contents	0.85 μg QCE/ g dry weight

2.2 Microwave-Assisted Extraction

Microwave have been used since World War II and the domestic ovens was the first commercial application of microwaves following by the development of radar technology. The energy of microwaves is used as a source of heat in analytical laboratories started in the late 1970s (Hadkar et al., 2013). Microwaves are electromagnetic waves that made up of two oscillating perpendicular fields which are magnetic field and electrical field (Destandau et al., 2013). The microwave energy is send directly to the sample through interaction of molecular with electromagnetic field by transformations of electromagnetic energy into thermal energy (Veggi et al., 2013).

Microwave assisted extraction is a sustainable technology for the extraction of phenolic compounds in which microwaves separate the cellular matrix and releasing intracellular compounds (Mandal et al., 2006). It also have an ability to heat a matrix internally and externally without a thermal gradient (Mandal et al., 2006). The working principle of microwave heating process is energy transfer that occur by interacting with polar solvent such as water with two mechanisms which are ionic conduction and dipole rotation mechanisms (Zhang et al., 2018). Ionic conduction is the electrophoretic migration of ions when applied an electromagnetic field. Dipole rotation is arrangement of polar molecules that have dipole moments with electric field (Veggi et al., 2013). Polar molecules that have permanent dipole moment such as phenolic compounds and ionic solutions completely captivate the microwave energy and enhance the extraction process (Osorio-Tobón, 2020)

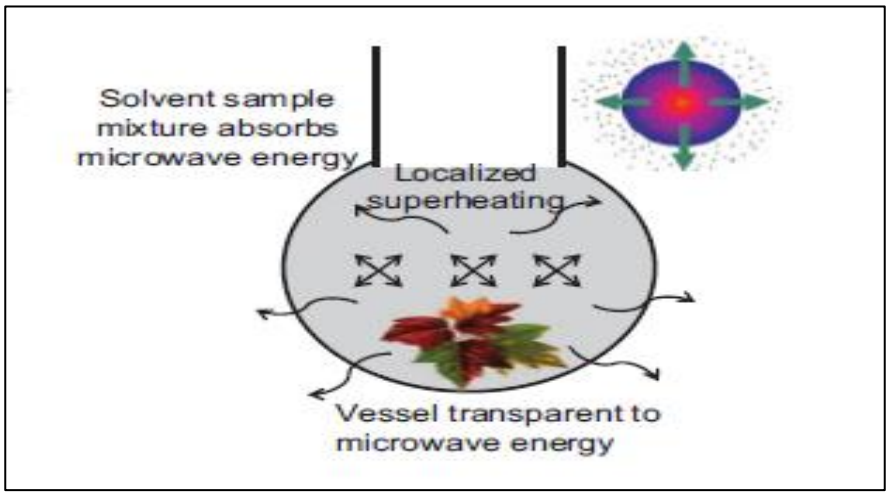

Figure 2.1 Microwave heating and temperature gradient by microwave energy (Destandau et al., 2013).

Figure 2.1 shows the microwave heats the whole sample simultaneously. Heating process occur in the targeted material with no heat being lost to the environment as it occurs in a closed system. Thus, this heating mechanism of advanced extraction method can reduce the extraction time compared to conventional technique.

The potential of the solvent to absorb the microwave energy and convert it as heat to the surrounding molecules depend on the dissipation factor (tan δ). The dissipation factor is given by the Equation (2.1) (Hadkar et al., 2013) :

$$\tan \delta = \frac{\varepsilon''}{\varepsilon'} \qquad (2.1)$$

Where, ε'' = dielectric loss (efficiency of converting microwave energy into heat)

ε' = dielectric constant (measure of the ability to absorb microwave energy)

The amount of absorption of microwave will be increased with the dielectric constant where the higher value of dielectric constant resulting higher efficiency of adsorption of microwave energy. Table 2.3 shows that the water solvent has highest value of dielectric constant which means that the energy from microwave being absorbed more by the system. When the absorption energy become higher, it will produce high temperature inside the materials will cause rupture of the cell. In order to prevent this phenomena to occur, the microwave power should be sufficient to reach the boiling point of water (Veggi et al., 2013) .

Table 2.3 Physical constants and dissipation factors for solvents used in MAE (Veggi et al., 2013)

Solvent	**ε' at 20°C**	**tan δ ($\mathbf{x10^{-4}}$)**	**Boiling point, (°C)**	**Viscosity, (cP)**
Water	78.3	1,570	100	0.89
Acetronitrile	37.5	-	82	
Methanol	32.6	6,400	65	0.54
Ethanol	24.3	2,500	78	0.69
Acetone	20.7	5,555	56	0.30
2-Propanol	19.9	6,700	82	0.30
Hexane	1.89	-	69	0.30

2.3 Comparison of Microwave-Assisted Extraction (MAE) with other Solid-Liquid Extraction Techniques

The advanced extraction technique has gained a great attention to the industries due to the efficient process with free of toxic solvents. Microwave-assisted has been acknowledged as highly potential and efficient technique compared to conventional

technique. Advanced method of extraction have emerged as an energy saving technologies for the extraction of bioactive compounds from plant materials.

Table 2.4 shows the comparison of conventional extraction method for *K. Parviflora* crude extracts with different value of total phenolic content. It can be concluded that different type of extraction method resulting different total phenolic content in *K. Parviflora* crude extract.

Table 2.5 presents the advantages and drawbacks of MAE compared to other extraction technique. Soxhlet extraction is a standard method commonly used in extraction of material which continuous solvent extraction method with a lengthy process involving 24 hours of extraction, large consumption of solvent and large particle size (Kramer & Ryan, 2000). The hazardous organic solvent used in the soxhlet extraction would bring acute effect toward our health and environment as well (Norfaezah et al., 2015). The limitation of this method dependent on particle size of sample as internal diffusion would be affected during extraction and evaporation temperatures would affect the quality of final products (Veggi et al., 2013). Ultrasound-assisted extraction stand out as a sustainable alternative which requires a moderate investment of solvent and energy. Furthermore, it is easy to operate, safe, economical and reproducible where this technology allow its development under condition of atmospheric pressure and at an ambient temperature (Medina-Torres et al., 2017). However, it will cause damage on the active constituents of medicinal plants through

formation of free radicals and consequently undesirable changes in the drug molecules occasionally due to the effect of ultrasound energy which is more than 20 kHz (Varma, 2016) . For the supercritical fluid extraction, chemical compound were extracted using supercritical carbon dioxide instead of an organic solvent because it is safe, nontoxic and affordable to buy it. However, even at high densities, carbon dioxide has a limited ability to dissolve highly polar compounds. This method more complex where many parameter to optimize especially analyte collection. Pressurized liquid extraction can be categorized as an alternative to soxhlet extraction where this technique allow the use of solvents or solvent mixtures with different polarities under high pressures (up to 20 MPa) that keeping the extraction solvent in the liquid state and temperature ranging from room temperature up to 200 °C. However, it can lead to the degradation of thermolabile analytes in order to pressurized solvent remains in the liquid state as well as allowing high temperature extraction (Veggi et al., 2013). In general, microwave-assisted extraction is more preferred in this study due to a shorter processing period, high quality of yield, low processing cost and wide capability to produce dried products (Ahmad et al., 2020).

Table 2.4 Comparison conventional extraction method of *K. Parviflora* crude extract based on total phenolic content

Extraction Type	**Total phenolic content**	**Reference**
Percolation (Ethanol 95%)	107 ±9.27 µg GAE/mg	Khaing et al., 2020
Percolation (Water 100%)	81.35± 0.20 µg GAE/mg	Khaing et al., 2020
Solvent extraction (Water)	74.3 mg GAE / g dry weight	Rahman et al., 2018
Solid phase extraction (Methanol: water: formic acid = 50:45:4, v/v/v)	43.88 mg/g dry weight	Asamenew et al., 2019
Solid-liquid extraction (Ethanol 100%)	141.59 ± 5.68 mg GAE/ 100 g dry weight	Misuna, 2017

Table 2.5 Comparison of different extraction method (Veggi et al., 2013)

Extraction Technique	Soxhlet	Microwave-assisted extraction (MAE)	Ultrasound-assisted extraction (UAE)	Supercritical fluid extraction (SFE)	Pressurized solvent extraction (PSE)
Description	• Sample is placed in a glass fiber thimble • Using Soxhlet extractor, sample repeatedly percolated with recondensed vapors of solvent	• Sample is immersed in a microwave-absorbing solvent in closed vessel and irradiated with microwave energy	• Sample is immersed in solvent in a vessel • Submitted it to ultrasonic using US probe or US bath	• Sample is loaded in high-pressure vessel and extracted with supercritical fluid (most commonly carbon dioxide at pressure of 150-450 bar and temperatures of 40-150 °C) • The analyte are collected in a small volume of solvent, in separator or onto a solid – phase trap which is rinsed with solvent in a subsequent step	• Sample and solvent are heated and pressurized in an extraction vessel • When the extraction is finished, the extract will automatically transferred in to a vial
Extraction time	3-48 hr	3-30 min	10-60 min	10-60 min	5-30 min
Sample size	1-30 g	1-10 g	1-30 g	1-5 g	1-30 g
Solvent use	100-500 ml	10-40 ml	30-200 ml	2-5 ml (solid trap) 5-20 ml (liquid trap)	10-100 ml
Investment	Low	Moderate	Low	High	High
Advantages	Easy to handle , no filtration necessary, high matrix capacity	Fast and multiple extraction, easy to handle, moderate solvent consumption, elevated temperatures	Easy to use, multiple extraction	Fast extraction, low solvent consumption, concentration of the extract, no filtration necessary, possible high selectivity, low temperature , no use of toxic solvents	Fast extraction, no filtration necessary, low solvent consumption, elevated temperature, automated system
Disadvantages	Long extraction time, large solvent volume, clean up step is needed	Extraction solvent must absorb energy, filtration step required, waiting time for the vessels cool down.	Large solvent volume, filtration step required, repeated extraction may be required.	Many parameter to optimize especially analyte collection.	Possible degradation of thermolabile analytes, clean up step is needed.

2.4 Important parameter in microwave-assisted extraction (MAE)

The effectiveness of the extraction process is correlated to the operating condition. The parameter that influence the extraction technique are microwave power, solvent to feed ratio and extraction time. Understanding the consequences and interaction of the factor on the MAE process is essential. Hence, the parameter that affect MAE process will be presented as a guideline to select right operation condition.

2.4.1 Microwave Power

The power of microwave should be chosen wisely in order to reduce the time to obtain the set temperature without reach the higher temperature and overpressure in a closed vessel. In a closed system, the maximum power range can be used between 600 W and 1000 W (Destandau et al., 2013). However, using high microwave power will lead to the poor extraction yield due to the thermal degradation of the compound and rapid rupture of the cell wall (Veggi et al., 2013). In microwave-assisted extraction of pitaya peels, microwave power were applied at different levels of power which at 200 W to 800 W. As a result, the optimum microwave power to obtain the maximum total phenolic content value (TPC) was 400 W. This finding is similar on extraction of orange peel where the result showed that the active compound of pectin increased with higher power and slightly decreased when the power applied was over 400 W (Nazeri & Zain, 2018). Thus, selection of microwave power on *K. Parviflora* crude extract should be in range 100 W to 600 W to prevent high pressure and temperature in the vessel.

2.4.2 Solvent to Feed Ratio

The solvent to solid feed ratio is an essential parameter that should be optimized. The solvent volume must be enough to ensure the entire sample is immersed in the

solvent so that the sample can swell during extraction (Destandau et al., 2013). Moreover, excessive of the extracting solvent will caused more energy and time required to condense the extraction solution in the final step and purification process (Veggi et al., 2013). The extraction process may lead to the lower recovery of the yield when higher ratio is used due to insufficient stirring of the solvent by microwave (Hadkar et al., 2013). In black mulberry fruit extraction, solvent to feed ratio were varied from 1:3 to 1:10 ratio. It was found that optimum condition of black mulberry fruit extract at 1:5.2 ratio (Ahmad et al., 2020). Thus, the solvent to feed ratio need to be in moderate ratio so that the recovery of the yield will be higher.

2.4.3 Extraction time

Extraction time is a key factor that determines energy consumption and process feasibility. Basically, when the extraction time is increasing, the quantity of analytes extracted will be increase which is more phenolic compound will be extracted (Hadkar et al., 2013). However, the possibility of oxidation of phenolic increase if reducing agent are added to the solvent system so that it can overcome the oxidation process (Naczk & Shahidi, 2006). The recommendation of the extraction time will be 15-20 minutes but even 40 second have shown excellent recovery for the extraction process (Hadkar et al., 2013). The study of microwave-assisted extraction of pectin from sour orange peel showed that the optimum conditions for the highest yield of pectin were obtained at irradiation time of 3 min where the microwave time was varied at 1 min to 3 min. In short, longer irradiation favour the production of pectin due to the time requirement for the exposure of the orange peel to release the biological compound from the raw materials (Hosseini et al., 2016).

2.5 Sustainability of Extraction Process

The sustainability of the extraction in this study consists of extraction method and type of solvent used. According to Jacotet-Navarro et al., 2016, the purpose of green extraction is to accomplish a faster extraction time, efficient energy consumption, increased mass and heat transfer and reduction of processing step (Soquetta et al., 2018). The idea of green chemistry was establish in 1991 during launching a programme of execution of sustainable development in chemical technology (Cvjetko Bubalo et al., 2018). This concept was based on 12 principles as a recommendation used to design chemical products and reduce hazardous substance that can harmful to human health and environment (Cvjetko Bubalo et al., 2018).

The theory of green extraction of natural product have been introduced by Chemat et al.,2012 on the basis of green chemistry and green engineering to modern sustainable process. Based on the definition of green extraction, the research and designing the extraction process that produced lower energy consumption, using alternative solvent and renewable natural products which the product safety and high quality of the extract or product obtained (Chemat et al., 2012, 2020).

The natural health products should be guided by the six principles of green extraction for industry and researches as a guidelines to create revolution in all feature of solid-liquid extraction. In this study of extraction, only two of the principle focused on which are **Principle 2** : Usage of alternative solvents and principally water or agro-solvents and **Principle 3**: Reduction of energy consumption by energy recovery and using innovative technologies (Chemat et al., 2012).

The alternative solvents that suitable for extraction should have high solvency and flash points with low toxicity and low environmental impacts, easily biodegradable and should be easy to recycle without any effect to the environment (Chemat et al.,

2020). Thus, the selection of solvent is essential in order to obtain higher yield of extract. Ethanol and water are type of green solvent can be used. Ethanol is bio-solvent that can be obtained by fermentation of sugar-rich material such as cereals and sugar-beet. However, ethanol is flammable and potentially explosive when used in a large scale even though it easily available due to the high purity, low price and biodegradable (Chemat et al., 2012). Thus, ethanol solvent is not preferable in this study due to the safety aspect. Besides, water can be considered as a greenest solvent due to the physicochemical changes of water from ambient to near critical conditions. Moreover, it also non-toxic to health, less pollution towards environment and least expensive solvent (Castro-Puyana et al., 2017). The high dielectric constant of water make it highly polar solvent because the dipole orientations in the H-bon network disintegrate when high temperature and pressures applied (Petigny et al., 2014). Hence, using water as an alternative solvent for extraction can reduced environmental impact, harmless to health and ease the process step.

2.6 Response Surface Methodology (RSM)

Response surface methodology (RSM) is a group of mathematical and statistical technique which an optimization mechanism that can recognize interrelationship between variables as being acquire by experiment or research studies in food and herbal plants extraction area. This method develops an appropriate experimental design that integrates all of the independent variables and input data that will be used for experiment which provides a set of equations that can give theoretical value of yield. The yield are acquired based on the controlled values of independent variables where the response can be predicted on the new values of independent variables (Said et al., 2015). The main objective for RSM is to investigate the optimum response and analyze

how the response changes by modifying the design variables (Bradley, 2013). By establishing RSM method in the optimization process, it can make the experiment or research study more efficient which short period of time is required.

On the other hand, Central Composite (CC) design or in short CCD is model under response surface that consists of three type of design which are circumscribed (CCC), inscribed (CCI) and face-centered (CCF). For CCD, α where the distance between the center point and star point is greater than 1 (Ranade & Thiagarajan, 2017). By using CCD, it allow the creator of experimental to recognize the effects of factor whether it goes beyond or below the chosen levels of factors and it provides high quality predictions of linear and quadratic interaction of parameters (Olawoye, 2020).

In this study, the low and high limit of independent variables which are microwave power (140 W and 511 W), microwave time (1 min and 3 min) and solvent to feed ratio (5:1 and 10:1). As for coded level of 0 (center), for all parameters are 364 W, 2 min and 7.5:1 ratio. The number of experimental, *N* to be run for CCD can be calculated by the given Equation (2.2) (Olawoye, 2020).

$$N = 2^n + 2n + nc \qquad (2.2)$$

where, N = number of experimental

n = number of factors or variables

nc = number of center points

CHAPTER 3

METHODOLOGY

3.1 Material

K. Parviflora rhizome was obtained from Bukit Merah, Perak. The sample was washed with water and chopped into fine pieces. Chemical reagent for phenolic content analysis is Folin-Ciocalteu phenol reagent which obtained from Merck KGaA and sodium carbonate. Gallic acid and ascorbic acid were obtained from Sigma-Aldrich (M) Sdn. Bhd, Malaysia and distilled water will be used as a extraction solvent in MAE. 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay was used to determine the antioxidant activity of the sample.

3.2 Instrumentation and Equipment

Table 3.1 shows list of instrumentation and equipment that used with their usage in extraction for experiment purpose.

Table 3.1 List of instrumentation and equipment

Equipment	Brand	Model	Usage
Centrifuge	Eppendorf	5702 R	Isolate liquid and solid
Electronic balance	A&D	GF-300	Weighing the sample
Freeze dryer	Labconco	FreeZone 12 L	Solvent removal
Freezer	Sano	VIP Series	Freeze supernatant
Knife	-	-	Chopped the rhizome finely
Micropipette	BrandTech	-	Transport small volume of liquid
Microwave	Electrolux	-	Extraction
Pestle and mortar	-	-	Grind dried rhizome
Spectrophotometer	Thermo Scientific	Genesys 20	Absorbance assay

3.3 Experimental flow chart

Figure 3.1 shows the flow of activity of the experiment to achieve the objective of the study.

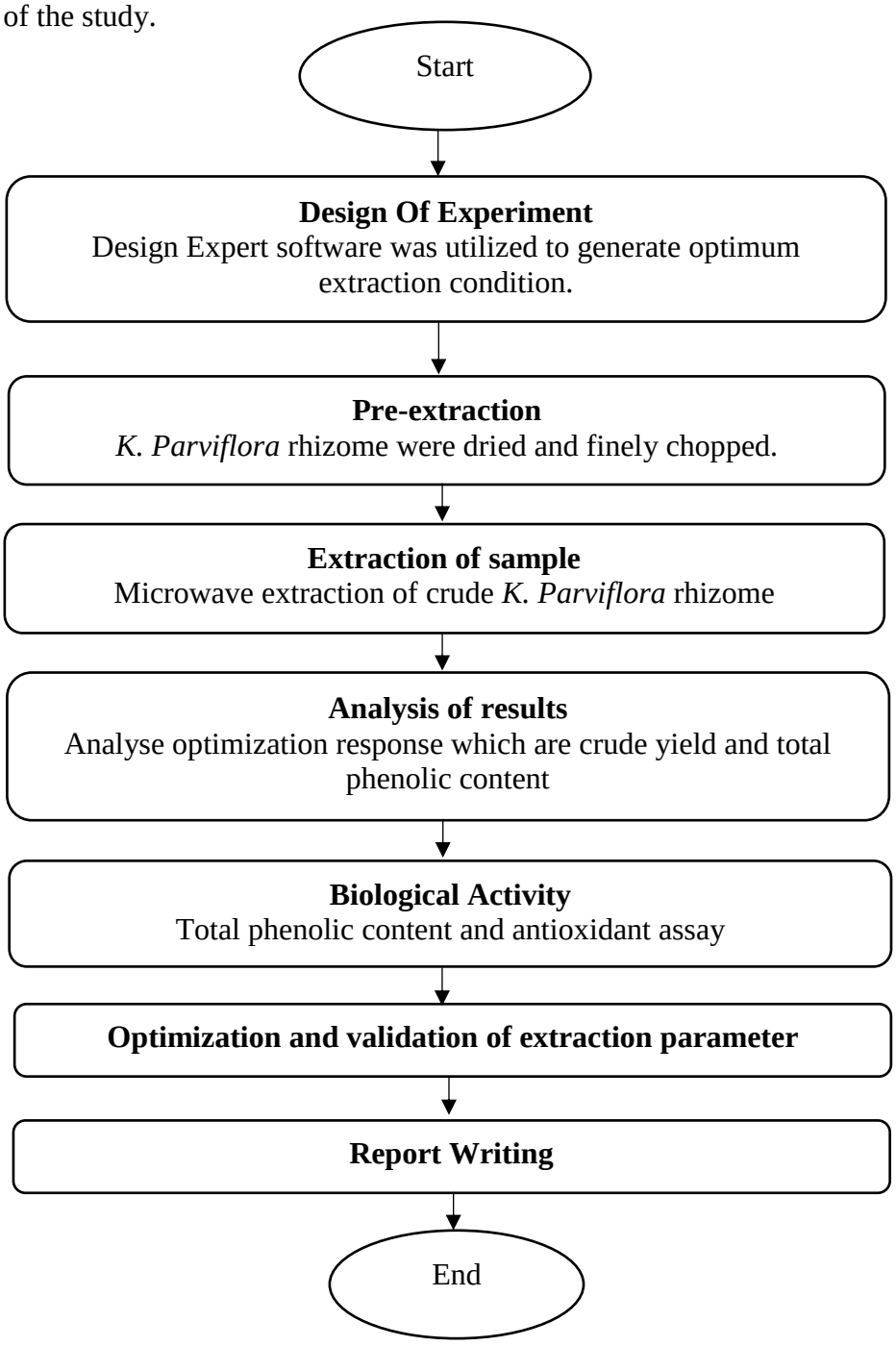

Figure 3.1 Flow diagram of research activity

3.4 Design of Experiment (DOE)

Design Expert 12 software was used to generate optimum extraction condition by response surface methodology (RSM) for the analysis of the raw data. The manipulated variable such as microwave power (W), microwave time (min), and solvent to feed ratio have been utilised using the software to enhance the efficacy of each factor during the experiment. The yield of extract, total phenolic content (TPC) and DPPH scavenging activity for the antioxidant were assigned as response analysis for this optimization. Response surface plot was used to demonstrate the relationship between the response and manipulated variable.

Table 3.2 shows the ranges of independent variables with a complete design matrix of experiment. The number of experimental obtained at each number of factors where number of variables, n = 3 and number of replicates for the centre point, nc = 6 which calculated using Equation (2.2). The total number of experiments, N which is 20 experiments were shown in the Table 3.3.

$$N = 2^3 + 2(3) + 6 = 20 \qquad (3.1)$$

Table 3.2 Coded and actual value of factors and their levels

Independent Variables		**Coded Level**		
Name	**Code**	-1	0	1
		Actual Levels		
Microwave power (W)	A	140	360	511
Microwave Time (min)	B	1	2	3
Solvent to Feed ratio	C	5	7.5	10

Table 3.3 Experimental design matrices for microwave-assisted extraction *of K. Parviflora* crude extracts

Std	Run	Factor 1 A: Microwave Power (Watt)	Factor 2 B: Microwave Time (min)	Factor 3 C: Solvent to Feed ratio
1	14	140	1	5:1
2	19	511	1	5:1
3	12	140	2	10:1
4	7	511	3	5:1
5	5	140	1	10:1
6	4	511	1	10:1
7	2	140	3	10:1
8	13	511	3	10:1
9	11	140	2	7.5:1
10	16	511	2	7.5:1
11	10	364	1	7.5:1
12	17	364	1	7.5:1
13	8	364	2	5:01
14	6	364	1	5:01
15	18	364	2	7.5:1
16	3	364	3	10:1
17	9	364	2	10:1
18	15	364	1	10:1
19	20	364	3	5:1
20	1	364	1	7.5:1

3.5 Pre –extraction

The rhizomes were weighed at 400 g using electronic balance and broken into pieces to expose the crevices then washed in running water to remove the adhering mud. The rhizomes were chopped into fine pieces after the rhizome were scrapped with a knife until the skin peeled off and were dried in the oven within eight hours with temperature up to 55 ℃ or dried at the open area which laid under the sun until the rhizome completely dried. The dried rhizomes were grinded using pestle and mortar to make it into powdery-like and stored in the dark, dry and at room temperature for the subsequent experimental works.

3.6 Extraction of *K. Parviflora* crude extract via Microwave-Assisted Extraction (MAE)

The biological compounds in *K. Parviflora* was extracted using distilled water as a solvent via microwave for the extraction process. 20 set of experiments were planned with ranges of the independent variables such as microwave power (140, 364,511 W), microwave time (1,2,3 min) and solvent to feed ratio (5:1, 7.5:1, 10:1). Parameter for the analysis of the response, the total phenolic content (TPC) and yield of crude extract was accomplished by using Folin-Ciocalteu reagent. DPPH scavenging activity was used to analysis the antioxidant activity. The gallic acid and sodium carbonate used as a standard of TPC analysis while ascorbic acid used as a standard of DPPH analysis by using spectrophotometer.

2 g of *K. Parviflora* powder sample were used with different distilled water ratio was then soaked in crucible. The crucible were placed in the microwave for the extraction process following the designated microwave power and time. Subsequently, the sample were allowed to cool down at room temperature. The supernatant was collected then placed in 50 ml centrifuge tubes and stored in refrigerator below 0°C for further analysis.

3.7 Post-extraction

The supernatant was separated from the residue solid by centrifugation at 2000 rpm for 10 min and then filtered through What-man filter grade 4. The crude extract was froze in the freezer below 0°C for at least 24 hour to turn the liquid phase into solid phase. The frozen extract then undergoes freeze dryer for maximum of 24 hr duration to ensure the sample extract completely dry and utilized for further analysis such as TPC, yield of extract and antioxidant activity.

3.8 Data collection and analysis

3.8.1 Determination of Yield of *K. Parviflora* crude extract

The yield of dried extract of *K. Parviflora* was determined based on the calculation of the dried extract obtained after freeze drying process and the amount of powdered rhizome that used during early stage of extraction in the microwave process which is 2 g. The calculation of yield was evaluated in percentage. Equation (3.2) shows the equation to determine the yield of *K. Parviflora* crude extracts

$$Yield\ of\ extract\ (\%) = \frac{W_f}{W_0}\ x\ 100 \qquad (3.2)$$

where :

W_f is the weight of dried extract after freeze drying process (g)

W_0 is the weight of powdered rhizome used in the extraction (g)

3.8.2 Determination of Total Phenolic Content (TPC) of *K. Parviflora* crude extract

Total phenolic content of *K. Parviflora* extracts were determined by the Folin-Ciocalteau (FC) method that measured using spectrophotometer with a slight modification that previously employed by Singleton and Rossi (1965). Gallic acid was used as a standard for calibration curve for the extract and TPC was expressed as milligram gallic acid equivalent per gram dry extract (mg GAE/g dry weight). For the TPC analysis, preparation of the three solution need to be done which is Folin-Ciocalteu reagent (FCR), 7% sodium carbonate Na_2CO_3 and blank solution.

GAE solution was used as a reference of TPC in *K. Parviflora* crude extract to obtain a calibration curve. The preparation of GAE solution as similar with the previous procedure but the extract was substituted with gallic acid. The solution was prepared by mixing 1 mg of Gallic acid (GA) with 1 ml of distilled water to get concentration of 1000 μg/ml or 1 mg/ml. 1 ml of different concentration of Gallic acid solution (0.12,