ANTIOXIDANT PROPERTIES OF TRADITIONAL CHINESE MEDICINAL HERBS (*Lycium barbarum* AND *Polygonum multiflorum*) WITH DIFFERENT PREPARATION METHODS

By

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ABSTRACT

ANTIOXIDANT PROPERTIES OF TRADITIONAL CHINESE MEDICINAL HERBS (*Lycium barbarum* AND *Polygonum multiflorum*) WITH DIFFERENT PREPARATION METHODS

Cheng Kah Mun

In this study, phytochemicals of two traditional Chinese medicinal herbs (*Lycium barbarum* and *Polygonum multiflorum*) were extracted using two different preparation methods, which were decoction and grounded methods. The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of each crude extract and their respective positive control (commercial extract pulveres of *L. barbarum* and *P. multiflorum*) were also examined quantitatively using the Folin-Ciocalteu reagent method, aluminium chloride colorimetric method, and DPPH scavenging activity assay, respectively. There were significant differences ($P < 0.05$) between the results obtained from the same herb prepared by different methods in TPC, TFC, and DPPH assay. The crude extract of *P. multiflorum* prepared by decoction method possessed the highest phenolic (70.21 ± 5.18 mg GAE/g DW) and flavonoids (168.57 ± 6.36 mg QE/g DW) contents among all the extracts. Results also showed that the crude extract of *P. multiflorum* prepared by decoction method exhibited the highest radical scavenging activity (EC_{50} = 24.22 ± 0.14 μg/mL) among all the extracts with references to ascorbic acid (EC_{50} = 9.29 ± 0.04 μg/mL). These

results indicated that different preparation methods affect the phenolic and flavonoid contents as well as antioxidant activity of herbs. Furthermore, EC_{50} showed a negative correlation with TPC and TFC, indicates that the phenolic and flavonoid contents were served as indicators of the antioxidant properties of the herbs. Thus, *P. multiflorum* and *L. barbarum* are good sources of antioxidants whereby *P. multiflorum* is recommended to be prepared by decoction method while *L. barbarum* should be prepared by grounded method to obtain the greatest antioxidant properties of the herbs.

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DECLARATION

I hereby declare that the final year project entitled "**ANTIOXIDANT PROPERTIES OF TRADITIONAL CHINESE MEDICINAL HERBS (*Lycium barbarum* AND *Polygonum multiflorum*) WITH DIFFERENT PREPARATION METHODS**" is based on my original work. I have not copied from any student's work or from any sources, except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(CHENG KAH MUN)

APPROVAL SHEET

This project entitled "**ANTIOXIDANT PROPERTIES OF TRADITIONAL CHINESE MEDICINAL HERBS (*Lycium barbarum* AND *Polygonum multiflorum*) WITH DIFFERENT PREPARATION METHODS**" was prepared by CHENG KAH MUN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Food Science at Universiti Tunku Abdul Rahman.

Approved by:

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Dr. Lye Huey Shi Date: _____

Supervisor

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PERMISSION SHEET

It is hereby certified that **CHENG KAH MUN** (ID No: **14ADB06235**) has completed this final year project entitled "ANTIOXIDANT PROPERTIES OF TRADITIONAL CHINESE MEDICINAL HERBS (*Lycium barbarum* AND *Polygonum multiflorum*) WITH DIFFERENT PREPARATION METHODS" under the supervision of Dr. Lye Huey Shi (Supervisor) from the Department of Agricultural and Food Science, Faculty of Science, and Dr. Teh Lai Kuan (co-Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(CHENG KAH MUN)

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LIST OF ABBREVIATIONS

℃	Degree celcius
2.4-DNPH	2,4-Dinitrophenylhydrazine
$ABTS^{\bullet+}$	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation
$AlCl_3$	Aluminium (III) chloride
ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C	Carbon
Ca^{2+}	Calcium ion
CAE	Catechin equivalent
Cu, Zn-superoxide dismutase	Copper, Zinc-superoxide dismutase
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazy
DW	Dry weight
EC_{50}	Effective concentration at which 50% of activity is observed
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant potential
g	Relative centrifugal force
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
GSH	Glutathione

HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration to reduce 50% of cell viability
kDa	Kilodalton
LBP	*L. barbarum* polysaccharide
MDA	Malondialdehyde
Na_2CO_3	Sodium carbonate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
PG	Propyl gallate
PPO	Polyphenol oxidase
QE	Quercetin Equivalent
r	Correlation Coefficient
ROS	Reactive oxygen species
SD	Standard Deviation
SOD	Superoxide dismutase
TCM	Traditional Chinese medicine
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TPC	Total phenolic content
TSG	Tetrahydroxystilbene glucoside
w/v	weight for volume

CHAPTER 1

INTRODUCTION

Free radicals that produced in human body from different physiological and biochemical processes are able to cause oxidative damage in biomolecules such as protein, lipids and DNA (Cai, et al., 2004). This damage will eventually leads to chronic diseases such as aging, diabetes, and cancer. Antioxidants have been reported to act as reducing agents to neutralize the free radicals. Antioxidants are classified into natural and synthetic antioxidants based on their sources (Fang, et al., 2002; Lobo, et al., 2010). Plants such as fruits, vegetables, and traditional medicinal herbs that contain phenolic compounds and other endogenous metabolites are examples of natural source of antioxidant for mankind. They are more cost effective and safe for consumption compared to propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and those synthetic antioxidants (Azlim Almey, et al., 2010).

In addition, numerous traditional Chinese medicinal herbs have been found to possess antioxidant properties due to high content of natural antioxidants. Some of the traditional Chinese medicinal herbs such as *Lycium* (*L*.) *barbarum* and *Polygonum* (*P*.) *multiflorum* have been proved to have high antioxidant properties by modern researcher due to the awareness on the rising use of them (Cai, et al., 2004; Li, et al., 2007; Preedy, 2014). Besides antioxidant properties, they also contain antidiabetic, anticancer, antimutagenecity, and other properties

(Cai, et al., 2004; Li, et al., 2007; Xutian, Zhang and Louise, 2009). These medicinal herbs have been practiced and systematically studied in pharmaceutical and dietary therapy for approximately 3000 years (Dong, 2013). All herbs have their own natures (cool, hot, warm and cold), flavours (pungent, sweet, sour, bitter and salty), and effects on corresponding meridian channels based on the "Yin and Yang" theory and "Qi" theory to promote optimal health outcome for mankind (Liao, Banbury and Leach, 2008; Xutian, et al., 2015).

Apart from that, there are different preparation methods of traditional Chinese medicinal (TCM) herbs available to promote the effectiveness and benefits of particular medicinal herbs. The herbs can be consumed as decoction, grounded powders, pills, tinctures, and other forms based on the instructions of Chinese medicine practitioner or consumer preference (Xutian, et al., 2015).

1.1 Background Information

Lycium barbarum and *Polygonum multiflorum* are two medicinal herbs that are generally used in the TCM preparation in Malaysia and commonly consumed by people in Malaysia especially Malaysian Chinese (Devalaraja, Jain and Yadav, 2011; Ahmad, et al., 2014; Adewoyin, et al., 2017). These two herbs have been documented to have antioxidant properties due to the presence of various phytochemicals and are being recognized as natural antioxidant sources by some researchers (Cai, et al., 2004; Li, et al., 2007; Zhang, et al., 2011; Preedy, 2014). Although various studies have been reported that *L. barbarum* and *P. multiflorum* possess antioxidant properties but there is no study on the effect of different preparation methods on their antioxidant properties prior to

consumption. Therefore, they were selected as samples in this research to examine the effect of different traditional Chinese medicinal herbs' preparation methods on the antioxidant properties of *L. barbarum* and *P. multiflorum* prior to consumption. Since the preparation methods of herbs were prior to consumption, water was used to extract the phytochemicals of the herbs based on different preparation methods and the crude extract was further proceed to determine the antioxidant activity.

1.2 Objectives

The objectives of this research were:

i. To extract phytochemicals from *L. barbarum* and *P. multiflorum* using two different preparation methods, which are decoction and grounded methods.

ii. To examine the total phenolics and flavonoids in *L. barbarum* and *P. multiflorum* extracts extracted from two different preparation methods.

iii. To determine the antioxidant activity of *L. barbarum* and *P. multiflorum* extracts extracted from two different preparation methods.

CHAPTER 2

LITERATURE REVIEW

2.1 Antioxidants

Antioxidants act as free radicals scavengers to hinder the damage or harmful effects caused by free radicals (Amzad Hossain and Shah, 2015). Free radicals are highly reactive molecules that consist of unpaired electrons which attack the healthy cells in human being and animals. They are mainly produced from environmental hazards such as air pollution, pesticides, smoking, and radiation (Kabel, 2014). In addition, they are also formed through breathing, physical activity, and other normal body functions (Poulson, et al., 1998; Lobo, et al., 2010). Overproduction of free radicals also damages the nucleic acids, lipids, and other cell structures (Fang, et al., 2002; Lobo, et al., 2010).

Generally, antioxidants can lose electron to free radicals rapidly and help in pairing the unpaired electron of latters. Several antioxidant mechanisms have been proposed to inhibit the oxidative damage caused by free radicals. Antioxidants can act as i) inhibitors to inhibit the formation of free lipid radicals and pro-oxidative enzymes such as lipoxygenases (Darmanyan, et al., 1998); ii) chain breaking antioxidants to disrupt the autoxidation chain reaction's propagation (Shahidi and Zhong, 2010); iii) as reducing agents to neutralize hydroperoxides (Shahidi and Zhong, 2010); iv) as metal chelators to stablize the

metal pro-oxidants (Saleh, et al., 2010); and v) as free radicals scavengers through synergism with other antioxidants (Carocho and Ferreira, 2013).

2.1.1 Exogenous and Endogenous Antioxidants

Antioxidants can be divided into exogenous and endogenous. Examples of endogenous antioxidants are glutathione peroxidase (GPx) and superoxide dismutase (SOD), which are enzymatic antioxidants, as well as glutathione (GSH) and other non-enzymatic antioxidants (Lobo, et al., 2010). They are participated in eliminating the excess reactive oxygen species (ROS) or free radicals (Lobo, et al., 2010). However, endogenous antioxidants that produced in human body are not sufficient to protect human beings from free radicals damage. Thus, exogenous antioxidants are needed to increase the protection of biomolecules in human body from free radicals and aid endogenous antioxidants in combating diseases (Amzad Hossain and Shah, 2015). Exogenous antioxidants are antioxidants that cannot be produced by human body naturally but usually present in particular food such as fruits and vegetables (Carocho and Ferreira, 2013). Polyphenols, carotenoids, vitamin C and vitamin E are exogenous antioxidants that can be obtained from the diets or supplements (Carocho and Ferreira, 2013).

2.2 Antioxidant Compounds in Medicinal Herbs

2.2.1 Natural Phenolic Compounds

Phenolic compounds are defined as a chemical compounds' class which made by an aromatic hydrocarbon group with a hydroxyl group bonded (Carocho and Ferreira, 2013). Phenol (C_6H_5OH), is the simplest structure chemical compound among this class and all other phenolic compounds are the derivatives of it. Most of the phenolic compounds are naturally occurred and widely used in manufacturing of perfumes and flavours due to their pleasant odours (Carocho and Ferreira, 2013; Amzad Hossain and Shah, 2015). Phenolic compounds are widely distributed in plants because they can be produced through plant metabolic system and aid in the growth of plants (Amzad Hossain and Shah, 2015). They are also used as natural antioxidants in processed foods and have potential against oxidative damage diseases due to their antioxidant activities (Amzad Hossain and Shah, 2015).

2.2.1.1 Flavonoids

Flavonoids are low molecular weight of phenolic compounds that have a common skeleton with diphenylpyrenes (C_6-C_3-C_6) (Procházková et al., 2011). They can be classified into six groups which are flavonols, flavanols, flavones, isoflavonoids, flavanones, and anthocyanins (Procházková et al., 2011). Their antioxidant properties are affected by the redox properties of their hydroxyl phenolic groups and the structural relationship in their own chemical structures (Carocho and Ferreira, 2013). Besides to act as reducing agents, they also function as metal chelators, superoxide radical scavengers, singlet oxygen

quenchers and hydrogen donators (Procházková, et al., 2011). They are involve in reducing α-tocopherol radicals, activating antioxidant enzymes, mitigating nitrosative stress, inhibiting oxidases, and increasing levels of uric acid, ascorbic acid, lipoic acid and other low molecular weight molecules (Rahal, et al., 2014). Quercetin, kaempferol, catechin, and catechin-gallate are some common examples of flavonoids (Procházková, et al., 2011). Figure 2.1 shows the base structure of flavonoids.

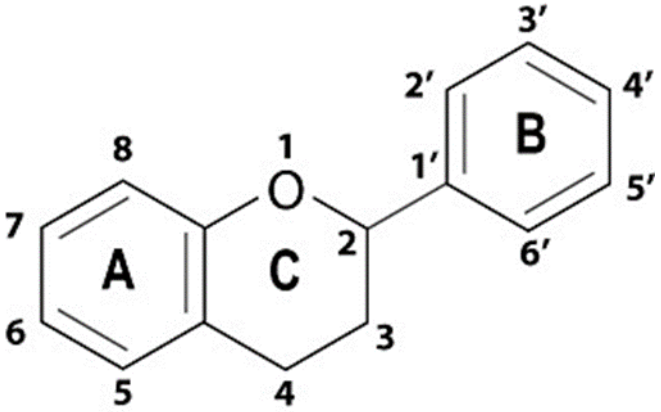

Figure 2.1: Base structure of flavonoid (Lillo, Lea and Ruoff, 2008)

2.2.1.2 Phenolic Acid

Phenolic acids are defined as aromatic secondary plant metabolites that usually present as esters and glycosides in plants (Terpinc, et al., 2011). They can be separated into hydroxycinnamic acid and hydroxybenzoic acid, due to their distinguish carbon frameworks (Terpinc, et al., 2011). They are able to terminate the attack of peroxyl and hydroxyl radicals, peroxynitrites, superoxide anions, and other free radicals, by acting as free radical scavengers and chelators in their antioxidant activities (Krimmel, et al., 2010). Gallic acid is an example of hydroxybenzoic acid whereas the hydroxyl derivatives of cinnamic acid is one of the examples of hydroxycinnamic acids (Krimmel, et al., 2010).

2.2.2 Carotenoids

Carotenoids are pigments that synthesized naturally by microorganisms and plants such as carrots, sweet potatoes, and tomatoes, but not found in animals and human beings. (Carocho and Ferreira, 2013). They can be divided into two groups. The first group is the carotenes, which are carotenoid hydrocarbons that consist of lycopene, β-carotene, and other specific end groups (Paiva and Russel, 1999). The second group is the xanthophylls, which are oxygenated carotenoids such as lutein and zeaxanthin (Paiva and Russel, 1999). In general, carotenoids are unreactive compounds but they may decompose to form non-radical compounds and bind to free radicals. This helps to reduce or discontinue the oxidative stress caused by free radicals (Carocho and Ferreira, 2013).

2.2.3 Vitamins and Minerals

Vitamins, for examples, vitamin C and vitamin E, are antioxidant compounds present in medicinal herbs. Vitamin C is known as ascorbic acid and composed of L-dehydroascorbic acid and L-ascorbic acid that responsible for antioxidant activity (Barros, et al., 2011). Meanwhile, vitamin E is known as tocopherols and composed of eight isoforms (Barros, et al., 2011). It is a lipid-soluble vitamin and a chain breaking antioxidant helps in protecting the integrity of lipid membranes (Barros, et al., 2011).

In addition, minerals play vital roles for numerous pathways in energy metabolism, usually present in a trace amount in medicinal herbs. Selenium and zinc are the most essential minerals in antioxidant activity (Tabassum, Bristow and Venkateswaran, 2010). They can prevent the formation of free radicals, rather than attacking or scavenging the free radicals (Tabassum, Bristow and Venkateswaran, 2010).

2.3 Traditional Chinese Medicinal Herbs

2.3.1 *Lycium barbarum*

2.3.1.1 General Description

Lycium barbarum, is a native deciduous shrubs with bright red berries (Kulczyńsk and Gramza-Michałowska, 2016). The fruit of *L. barbarum*, an ellipsoid orange-red berry approximately 1 to 3 cm long with sweet and tangy flavours (Bucheli, et al., 2011a). It is well known as ‘goji berry’ or ‘Chinese

wolfberry' (Kulczyńsk and Gramza-Michałowska, 2016). It plays a role in TCM for over 2000 years and commonly naming as 'gou ji zhi' in Chinese (Bucheli, et al., 2011a).

Lycium barbarum has oblong-lanceolate leaves with a maximum length of 7 cm long and 3.5 cm wide (Bucheli, et al., 2011a). It also has purple coloured flowers during flowering. Its flowering period takes place from June to September and the maturation of its fruits begin from August to October (Bucheli, et al., 2011a). However, the growth of *L.barbarum* is highly depends on latitude, altitude, and climate of the grown area (Bucheli, et al., 2011a). The seeds of *L.barbarum* are small, yellow and with a number of 4 to 60, inside the mature fruits (Bucheli, et al., 2011a). Figure 2.2 shows the *L. barbarum* before harvested whereas Figure 2.3 shows the dried fruits of *L. barbarum* which frequently used as TCM herbs.

2.3.1.2 Taxonomical Classification

Lycium barbarum was named by Carl Linnaeus in 1753 (Kulczyńsk and Gramza-Michałowska, 2016). It is a plant belongs to the *Planta*e kingdom, which is the first level in its taxonomical hierarchy, down to *Tracheobionta* subkingdom, *Spermatophyta* superdivision, and *Magnoliophyta* division. *Magnoliophyta* means flowering plants. Under this division, it is further categorised into class of *Magnoliopsida* and subclass of *Asteridae*. It is then further down to the order of *Solanales* and in the family of *Solanaceae* (United States Department of Agriculture, 2017a).

Figure 2.2: *Lycium barbarum* before harvested (Bucheli, et al., 2011a)

Figure 2.3: Dried fruits of *Lycium barbarum* (Goji berries) (Bucheli, et al., 2011a)

2.3.1.3 Habitat and Distribution

Lycium barbarum are native to Asia, primarily in a small autonomous region located at central north region of China, which named Ningxia (Bucheli, et al., 2011a). Nowadays, in China, there are also commercial volumes of *L. barbarum* grown in Xinjiang, Shaanxi, Hebei, Inner Mongolia, and Gansu (Kulczyńsk and Gramza-Michałowska, 2016). They are also widely grown in Japan, Korea, and Taiwan. *Lycium barbarum* had been traded since ancient times due to their medicinal and nutritious value and were imported to Europe in the 18th century. They are also cultivated in Europe (Bucheli, et al., 2011a; Kulczyńsk and Gramza-Michałowska, 2016). They require lots of light to grow but can also expose to partial shade. Besides, they are well-adapted to both warm and cold climates. They are blooming in summer and growing well on calcium-enriched dry sand (Bucheli, et al., 2011a).

2.3.1.4 Constituents and Phytochemicals

Lycium barbarum consists of many phytoconstituents such as polysaccharides, carotenoids, phenylpropanoids, and others (Kulczyńsk and Gramza-Michałowska, 2016). Polysaccharides are the most abundant and vital group of compounds in *L. barbarum* (Kulczyńsk and Gramza-Michałowska, 2016). These polysaccharides are water soluble and comprised of 5 to 8% of the total dry matter of the fruits with molecular weight ranged from 8 to 214 kDa (Amagase and Farnsworth, 2011).

The second highly significant group of phytochemicals that found in *L. barbarum* fruits is carotenoids (Kulczyńsk and Gramza-Michałowska, 2016). Carotenoids are comprised of 0.03 to 0.5% of dry matter of the fruits and responsible for the characteristic orange-red colour of the fruits (Kulczyńsk and Gramza-Michałowska, 2016). Zeaxanthin, also found in the form of dipalmitin zeaxanthin, is the most common carotenoids in *L. barbarum* fruits (Kulczyńsk and Gramza-Michałowska, 2016). Besides zeaxanthin, *L. barbarum* fruits also contain low amounts of beta-carotene, neoxanthin, and cryptoxanthin (Peng, et al., 2005; Wang, et al., 2010).

Furthermore, a high antioxidant capacity of phenylpropanoids is also detected in the fruits. Based on the study of Guo, et al., (2008), the presence of phenylpropanoids has been confirmed at the level of 22.7 mg (gallic acid equivalent/g extract). Other confirmed constituents are phenolic compounds (flavonoids), vitamins (thiamine and vitamin C), free amino acids (proline and taurine), minerals (potassium and sodium), organic acids (citric acid and malic acid), and fatty acids (palmitic and linoleic acids) (Potterat, 2009; Llorent-Mart ńez, et al., 2013; Donno, et al., 2015).

2.3.1.5 Traditional and Modern Uses of *L. barbarum*

In TCM, *L. barbarum* is primarily acts on the liver, lung, and kidney channels. They are found to enhance immune system function, protect liver, kidney and heart, improve blood circulation, aid in eyesight, boost fertility and have many other health benefits (Bucheli, et al., 2011a; Kulczyńsk and Gramza-

Michałowska, 2016). The recommended dosage of *L. barbarum* is 6 to 15 g in TCM preparations, taken twice or thrice daily (Bucheli, et al., 2011a). It is commonly used for treating "Yin" deficiency and "Qi" stagnation in liver and kidney for medicinal purposes (Liu and Tseng, 2005).

Traditionally, dried *L. barbarum* fruits are cooked before consumption. They can be used as herbal teas, to make Chinese soup with the combination of meat and other herbs, and can be added into various meat and vegetarian dishes (Bucheli, et al., 2011a). Other than that, they are also used in the production of wine and tincture (Amagase and Farnsworth, 2011). They can be eaten raw or consumed fresh (Amagase and Farnsworth, 2011). In modern days, they are widely used in production of juice and beverage (Amagase and Farnsworth, 2011). In the Western world, they act as functional foods or healthy supplements and are used to enhance flavour of some conventional food products such as yoghurts (Bucheli, et al., 2011a).

2.3.1.6 *In vitro* Studies of Antioxidant Effect of *L. barbarum*

Previous studies done by Peng, et al., (2005) and Guo, et al., (2008) showed that *L. barbarum* fruits exhibited a high antioxidant activity by scavenging the free radicals. Its polysaccharides complexes, flavonoids, carotenoid pigments, and vitamins such as ascorbic acid are constitutes that responsible for its high antioxidant activity (Wang, et al., 2010; Amagase and Farnsworth, 2011). However, there is limited study on the antioxidant properties of *L. barbarum* with different TCM preparation methods.

Several *in vitro* studies have proved the antioxidant activity of *L. barbarum* using several methods are summarised and presented in Table 2.1.

Table 2.1: Antioxidant activity of *L. barbarum* using several methods in previous studies

References	TEAC	FRAP	DPPH	TPC
Medina (2011)	-	-	-	895 [mg GAE/100g]
Henning, et al. (2014)	2.7 [mmol TE/100g]	0.9 [mmol TE/100g]	1.4 [mmol TE/100g]	-
Mocan, et al. (2014)	35.72 [μg TE/mg]	-	29.30 [μg QE/mg]	6159 [mg GAE/g]
Reis, et al. (2014)	-	-	-	15.67 [mg GAE/g]
Donno, et al. (2015)	-	-	-	268.35 [mg GAE/100g]
Asănică, et al. (2016)				8.95 [mM GAE / ml juice]
Zhang, et al. (2016)			85 [μM TE/g]	73.4 [mg GAE/g]

Note: TEAC: Trolox Equivalent Antioxidant Capacity; FRAP: Ferric Reducing Antioxidant Potential; DPPH: 2,2'-diphenyl-1-picrylhydrazyl; TPC: Total Polyphenol Content; QE: Quercetin equivalents; TE: Trolox equivalents; GAE: Gallic Acid Equivalents

2.3.1.7 *In vivo* Studies of Antioxidant Effect of *L. barbarum*

A study of Wu, Ng and Lin, (2004) reported the *L. barbarum* extract exhibited antioxidant activities via superoxide anion scavenging activities and showed a strong inhibition on malondialdehyde (MDA) formation in rat liver. A similar observation was reported by Cui, et al., (2011), where *L. barbarum* extract showed an antioxidant effect by reducing the oxidative stress in high-fat-diet mice's liver. Results also revealed that *L. barbarum* increased the antioxidant enzyme activities and concentrations of glutathione, and the MDA level in those rats. Meanwhile, Cheng and Kong (2011) found that the *L. barbarum* extract showed a protective effect on alcohol-induced liver necrosis rats by reducing the activity of liver damage biomarkers.

Other than that, a previous study of Li, et al. (2007) analysed the effect of *L. barbarum* polysaccharide (LBP) on oxidative stress of mice caused by ageing. An increased activity of endogenous antioxidant system, a decreased MDA level and an increased total antioxidant capacity in the liver, lungs, heart, and brain were observed in mice fed with LBP. Then, Shan, et al. (2011) also found that LBP was able to reduce the oxidative stress caused by physical activity on rats, as attributed by decreased MDA contents and increased levels of SOD and GPx. An increased in antioxidant enzymes activity and a decreased lipid peroxidation were also observed in humans upon consumption of *L. barbarum* or Goji juice (Amagase, et al., 2009; Bucheli et al. 2011b).

2.3.2 *Polygonum multiflorum*

2.3.2.1 General Description

Polygonum multiflorum has various common names such as Fo-Ti, *Fallopia multiflora*, Kashuu, Chinese knotweed, flowery knotweed, and fleeceflower root (Puri, 2011). Its Chinese name is He-Shou-Wu, with a legend that said it returned a man whose surname is He from previously grey-haired to natural black hair colour (Preedy, 2014). There is another legend said that He-Shou-Wu, was the name of a Tang dynasty man who was cured from infertility by this herb (Puri, 2011). It has a long history of keeps one strong and healthy and help to prevent the aging's effects (Puri, 2011).

It is classified as vine plants that are able to grow to about 4 to 5 m high in a shady bed (Preedy, 2014). It has spear-head shaped leaves and its flowering occurs from September to October (Preedy, 2014). During flowering, it has hermaphrodite white flowers that pollinated by insects (Puri, 2011). The root is tuberous and the root tuber of *P. multiflorum* is usually dug or harvested in spring and autumn, generally from a 3 to 4 years old plant (Puri, 2011). The reddish-brown root tuber is then washed, cut into slices, and dried under the sun prior to be used as TCM herbs (Preedy, 2014). Figure 2.4 shows the *P. multiflorum* before harvested and the Figure 2.5 shows the dried roots of *P. multiflorum* which are used as TCM herbs.

Figure 2.4: The *Polygonum multiflorum* plants (Lin, et al., 2015)

Figure 2.5: Dried roots of *Polygonum multiflorum* (Lin, et al., 2015)

2.3.2.2 Taxonomical Classification

Polygonum multiflorum is a plant belongs to the *Planta*e kingdom, which is the first level in its taxonomical hierarchy, down to *Tracheobionta* subkingdom, *Spermatophyta* superdivision, and *Magnoliophyta* division. *Magnoliophyta* means flowering plants. Under this division, it is further categorised into class of *Magnoliopsida* and subclass of *Caryophyllidae*. Then, it is further divided into the order of *Polygonales* and in the family of *Polygonaceae* (United States Department of Agriculture, 2017b).

2.3.2.3 Habitat and Distribution

The root tuber of *P. multiflorum* is a perennial climber, and native to East Asia, such as China, Japan, Taiwan, and Malaysia (Preedy, 2014). It grows in woods, along the bank of the stream, and in valley. It prefer light and sandy environment and requires moist and loamy soil (Preedy, 2014). It can exposed to semi-shade or sun (Puri, 2011).

2.3.2.4 Constituents and Phytochemicals

The antioxidant components such as 2, 3, 5, 4'-tetrahydroxystilbene 2-O-beta-D-glucopyranoside, catechin, and gallic acid have been identified by Chen, et al. (1999) from *P. multiflorum*'s root. Then, several compounds such as gallic acid, emodin, rhein, and an unknown glycoside have been isolated from *P. multiflorum*'s root extract using high speed counter-current chromatography (Yao, et al., 2006). Later, a new stilbenoid, (E)-2,3,4',5-β-tetrahydroxystilbene-

2-β-D-glucopyranoside was also identified from the root extract (Kim, et al., 2008). Scientific studies have confirmed that *P. multiflorum* contains some antioxidant compounds (phenolic compounds and flavonoids) and its major constituents include polygonimitins, chrysophanol, emodin, anthraquinone, phospholipids, tannins, and tetrahydroxystilbene glucoside (Puri, 2011; Feng and Bounda, 2015).

2.3.2.5 Traditional and Modern Uses

Polygonum multiflorum is a renowned "longevity herb" in TCM to help in developing "Qi" and treating "Yin" deficiency (Feng and Bounda, 2015). The herbal remedy of this herb is prescribed to enhance general health and greater longevity (Puri, 2011). Since *P.multiflorum* is always used as a kidney and liver tonic in TCM, it is said to be able to return grey hair into the original dark colour. This is because in Chinese medicine theory, the kidneys control the growth and colour of hair (Feng and Bounda, 2015). *Polygonum multiflorum* is commonly used as a blood toner and functioned in strengthening bones, muscles, and tendons (Feng and Bounda, 2015). It is also used to treat vaginal discharges, angina pectoris, impotence, weakness, and premature aging in TCM (Balch, 2012). Moreover, it can be used orally for hyperlipidaemia, lower back and knee soreness, limb numbness, insomnia, and dizziness with tinnitus (Feng and Bounda, 2015). Furthermore, it is also used for treating lymph node tuberculosis, constipation, and even cancer (Puri, 2011).

In addition, it has sweet and bitter taste, slightly astringent and has warm properties in TCM. Its recommended dosage in powder, decoction, medicated wine, oil, or pills is from 10 to 20 g (Puri, 2011). Overconsumption of *P.multiflorum* may cause side effects or adverse reactions such as numbness in the arms or legs (Puri, 2011). Apart from that, older *P.multiflorum*'s roots are in greater demands compared to younger roots due to higher effectiveness in treating diseases as emphasised by the traditional Chinese herbalists (Feng and Bounda, 2015). Besides oral consumption of *P. multiflorum*, it can also applied on the body surface to treat inflammations, carbuncles, itching, and skin eruptions (Feng and Bounda, 2015).

Traditionally, the root of *P. multiflurom* is often processed, cured or cooked prior to use (Feng and Bounda, 2015). One of the common processed methods is steaming the root for 12 h and sun drying for 8 h, and the processes usually repeated up to nine times (Lin, et al., 2015). The unprocessed or raw root is sporadically used (Feng and Bounda, 2015). According to TCM, it is believed that if it boiled in a special black beans' liquid, it is considered a fairly different and superior medicine (Lin, et al., 2015). It is usually consumed orally in tea form, decoctions, tinctures, and powder form (Feng and Bounda, 2015). It can be consumed as a single herb or in combination with other herbs by following the instructions of TCM practitioners (Preedy, 2014). In addition, it can be manufactured into ointment or balm to be used topically (Feng and Bounda, 2015). Today, it is available in the market in numerous forms, such as supplements, pills, powder, extract, tea, capsules, oil, juice, shampoo, and Shou Wu Chih (a liquid tonic formula) (Puri, 2011).

2.3.2.6 *In vitro* Studies on Antioxidant Effect of *P. multiflorum*

Chen, et al. (1999) conducted a study to extract and analyse the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging active components from the root of *P. multiflorum*. The antioxidant components of the root were identified by an activity-directed purification and fractionation process. In the study, 95% ethanol was used to extract the dried root of *P. multiflorum* and then the extract was divided into water, hexane, and ethyl acetate fractions. Results showed that the ethyl acetate fraction exhibited the strongest antioxidant activity in the DPPH test among all the fractions. Three strong antioxidant compounds (2,3,5,4'-tetrahydroxystilbene 2-O-β-D-glucopyranoside, catechin, and gallic acid) were also identified by spectral methods.

Besides, Ryu, et al., (2002) found that the *P. multiflorum*'s root extract showed a significant antioxidant activity. The results proved that its stilbene glucoside compound was the active constituent that contributes to its antioxidant property and it exhibited a high DPPH radical scavenging activity in term of IC_{50} (0.38 microM).

Later, Lin, et al., (2010) investigated the antioxidant activities of fresh *P. multiflorum*'s leaf, stem and root. Results showed that the *P. multiflorum*'s root had the highest reducing power, nitric oxide (NO) scavenging ability, total antioxidant activity, and DPPH scavenging activity compared to stem and leaf. However, there is lack of information and study on the antioxidant properties of *P. multiflorum* prepared using different TCM preparation methods.

2.3.2.7 *In vivo* Studies on Antioxidant Effect of *P.multiflorum*

According to Chiu, et al. (2002), the antioxidant action of an anthraquinone-containing *P. multiflorum*'s root extract was compared with its active constituents, emodin in evaluating their impacts on the antioxidant status of hepatic mitochondrial glutathione in carbon tetrachloride intoxicated rats. Results showed that a stronger *in vivo* antioxidant action was produced by the *P. multiflorum*'s root extract compared to emodin by their efficacy to increase hepatic mitochondrial glutathione antioxidant status in mice.

Besides, Hwang, et al. (2006) studied the antioxidant activities and effects of *P. multiflorum* thumb extract on the skin damage caused by ultraviolet B irradiation. The *P. multiflorum* extract-treated group of mice showed an increased in the Cu, Zn-superoxide dismutase immunoreactivity in a dose-dependent manner. This indicates that *P. multiflorum* extract was able to inhibit the destruction of Cu, Zn-superoxide dismutase caused by ultraviolet radiation and might contain anti-skin photoaging agents.

Moreover, Zhou, et al. (2015) evaluated the protective effect of tetrahydroxystilbene glucoside (TSG) derived from *P. multiflorum* against aging process in mice. The mice fed with TSG showed an improved memory ability, reduced levels of ROS, NO, and insulin-like growth factor, and increased levels of SOD, Ca^{2+}, and Klotho protein.

2.4 Preparation Methods of TCM Herbs

2.4.1 Decoction Method

Decoction is the most common preparation method of TCM herbs. The main aim of decoction is to extract the therapeutic constituents of the herbs by using hot water (Balch, 2012). Since TCM herbs are usually tough or fibrous, the simplest way for individuals to consume them safely is to boil them up in water and drink the resulting liquid or consume the softened herbal materials that had been boiled for a long time (Balch, 2012). Some hard herbal materials are required boiling for at least 10 min and then simmer for a number of hours. Nevertheless, preparation of herbal decoctions is relatively rapid and easy compared to converting the herbs into powdered form (Luo, et al., 2012).

Moreover, the container used and the decoction time will directly affect the quality of the herbal decoction. The container used should be clay, ceramic or glass pot instead of metal pot in preparing herbal decoction (Balch, 2012). In addition, the decoction time depends on the properties of the herb, the dosage of the herb and the patients's clinical condition (Balch, 2012). Therefore, individuals must follow the instructions of Chinese medicine practitioners in preparing herbal decoctions (Balch, 2012).

2.4.2 Grounded Method

Besides, the TCM herbs also can be consumed in powdered form or further proceed to modern capsules, pills and tablets (Luo, et al., 2012). It is a laborious

process to convert the TCM herbs into powdered form (Luo, et al., 2012). Firstly, the herb must be reduced in size or cut into small pieces (Balch, 2012). Then, the herbs are allowed to dry sufficiently under the sun to make them brittle before ground into fine powder by human efforts (Balch, 2012). Mortar can be used to grind small amounts of herbs. Thus, it is not a preferable option for most individuals to convert the TCM herbs by themselves. However, they are willing to purchase the prepared powdered TCM herbs in local herbal stores due to convenience. These powders are usually prepared by the Chinese medicine practitioners in a traditional way or produced by machinery in the modern licensed medicine factories (Luo, et al., 2012). The powders can be consumed by boiling in water for a few minutes, or swallowing down directly in powdered form or capsules, pills, and tablets which were the further processed form of powdered herbs (Luo, et al., 2012). It is much more convenience for patients that have acute symptoms, debilitated or don't have time or materials to prepare the herbal decoctions (Luo et al., 2012). Furthermore, the advantages of powdered herbs include prevent wastage of precious materials, suitable for herbs that have poor extraction yields, and potential to lose potency with heat or evaporation (Balch, 2012).

2.4.3 Tinctures

Tinctures are liquid extract made by dissolving herbs in alcohol. Unlike water, alcohol serves a role as an herb and consists medicinal properties (Balch, 2012). Tinctures can be consumed only for specific illness and not suitable for all individuals due to its warming properties (Balch, 2012). Hence, it is not a

common preparation method of TCM herbs compared to decoction and grounded methods (Balch, 2012).

2.5 Antioxidant Assays

2.5.1 Total Phenolic Content Assay

Since phenolic compounds are the indicator of the medicinal herbs' antioxidant property, total phenolic content (TPC) assay was frequently used to examine the total phenolic compounds present in the tested samples. Most of the TPC assay carried out were based on Folin-Ciocalteu (FC) method. According to Agbor, Vinson and Donnelly (2014), the FC reagent is a mixture of phosphomolybdic acid and phosphotungstic acid. It will form a blue chromophore constituent when it receive electrons transferred from phenolic compounds in alkaline medium. The degree of the blue coloured complex is depends on the absorption of FC reagent and the concentration of phenolic compounds present in the samples (Agbor, Vinson and Donnelly, 2014). The blue coloured complex can be measured with a spectrophotometer in the range of 690 to 710 nm (Agbor, Vinson and Donnelly, 2014). A reference standard compound such as gallic acid is usually used and the results are expressed based on it, for instance, gallic acid equivalents (GAE) (Agbor, Vinson and Donnelly, 2014).

2.5.2 Total Flavonoid Content Assay

Flavonoids, which are the largest group of natural phenolic compounds, are also indicators for the antioxidant properties of medicinal herbs. Only flavonols and flavones will react with aluminium chloride to form a stable complex at 415 nm

whereas flavanonols and flavanones react better with 2,4-dinitrophenylhydrazine (2.4-DNPH) to produce hydrazones at 495 nm among four groups of flavonoids (Ud-Din, et al., 2009).

According to Kalita, et al. (2013), the most common method used to examine the total flavonoid content of samples is aluminium chloride method. In this assay, there is a reaction of C-4 keto group (C=O) or hydroxyl group (O-H) on either C-3 or C-5 of flavonols and flavones with the aluminium chloride to form orange or pink coloured acid stable complexes (Kalita, et al., 2013). Acid labile complexes also create when the ortho-dihydroxyl groups in the A- or B-ring of flavonoids react with aluminium chloride (Kalita, et al., 2013). Then, the resulting complexes can be measured at 415 nm (Kalita, et al., 2013). Standard reference materials such as quarcetin can be used to build a calibration curve and the results can be obtained based on it (Kalita, et al., 2013).

2.5.3 DPPH Radial Scavenging Activity Assay

DPPH is the abbreviation of 2,2-diphenyl-1-picrylhydrazyl, which is a stable free radical as a result of the delocalization of electrons all over the molecule (Molyneux, 2004). The DPPH assay is a common and rapid antioxidant assay based on the antioxidant's scavenging activity to free radicals. In this assay, antioxidants act as hydrogen donating compounds when they react with DPPH. The electrons of DPPH become paired off and the DPPH solution is discoloured from purple to pale yellow solution due to the formation of hydrazine or reduced DPPH (Kedare and Singh, 2011). According to Proestos, et al. (2013), the degree

of discoloration of DPPH depends on the free radical scavenging activity exhibited by the antioxidant compounds present in the samples. The colour changes can be measured at wavelength 517 nm to quantify the level of antioxidant (Proestos, et al., 2013).

Antioxidant activity of medicinal herbs determined using DPPH assay is generally expressed as EC_{50} value. EC_{50} is defined as the concentration of a sample or extract to produce 50% of a maximal drug effect and is commonly used to study the drug or medicinal effects *in vitro* (Baharum, et al., 2014). Figure 2.6 shows the principle and reduction process of DPPH radical scavenging activity assay.

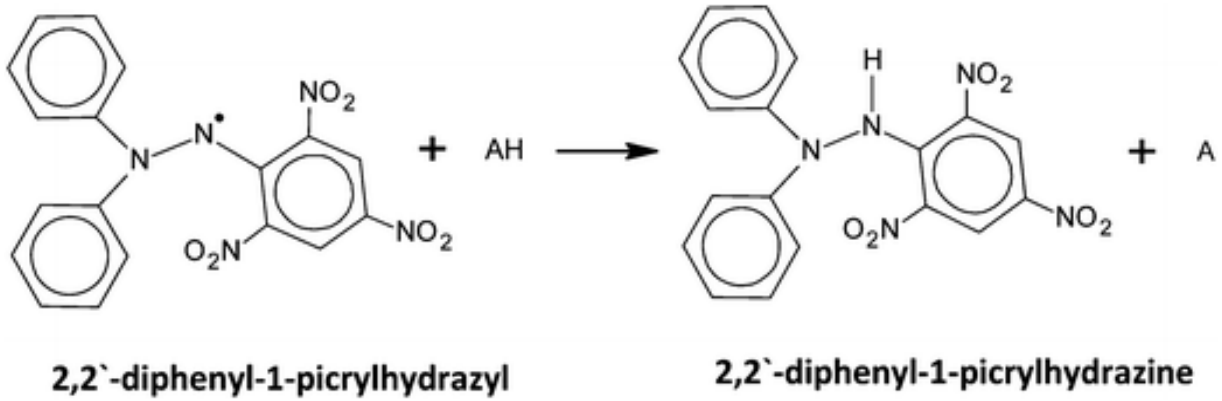

Figure 2.6: Principle of DPPH radical scavenging activity assay (Pyrzynska and Pękal, 2013)

CHAPTER 3

MATERIALS AND METHODS

3.1 Herb Materials

Dried herbs and powder forms of *L. barbarum* and *P. multiflorum* herbs were purchased from local Chinese medicine practitioners while two commercial extract pulveres of these two herbs manufactured by Sheng Foong company from Taiwan were purchased from a local herbal store and authenticated by Dr. Teh Lai Kuan, from Department of Biomedical Science, University Tunku Abdul Rahman. Commercial extract pulveres of *L. barbarum* and *P. multiflorum* were used as positive control in this study for comparison with the efficacy of the samples.

3.2 Chemicals

All chemicals used in this research were provided by Department of Agricultural and Food Science, University Tunku Abdul Rahman. The list of chemicals used and their respective manufacturer and country are shown in Table 3.1.

Table 3.1: List of chemicals used throughout the research.

Chemicals	**Manufacturer, Country**
Aluminium Chloride Anhydrous	Friendemann Schmidt, Australia
Ascorbis acid	Hmbg, Malaysia
DPPH (2,2-Diphenyl-1-picrylhydrazyl) reagent	Sigma Aldrich, U.S.A
Folin-Ciocalteau's phenol reagent	Merck, Germany
Gallic acid	R&M chemicals, U.K.
Methanol	QRëC™, Malaysia
Quercetin	Sigma Aldrich, U.S.A
Sodium carbonate	Merck, Germany
Sodium hydroxide	Merck, Germany
Sodium nitrite	Bendosen, Malaysia

3.3 Equipment and Laboratory Ware

The list of equipment and laboratory ware used in this research are shown in Table 3.2.

Table 3.2: List of equipment and laboratory ware used throughout the research.

Equipment/ Laboratory ware	**Company, country**
Centrifuge machine	Sigma, Germany
Drying oven	Binder, Germany
Electronic balance	Mettler Toledo, Switzerland
Freezer (-20 ℃)	Liebherr, Germany
Hotplate	Stuart, UK
Micropipette	Gilson, U.S.A
Microplate reader	BMG Labtech, Germany
Rotary evaporator	R-200 Buchi, Swirtzerland
Shaking incubator	Infost HT, Switzerland
Vortex mIxer	Scientific Industries, U.S.A

3.4 Overview of Methodology

A brief workflow of the methodology of this research is shown in Figure 3.1.

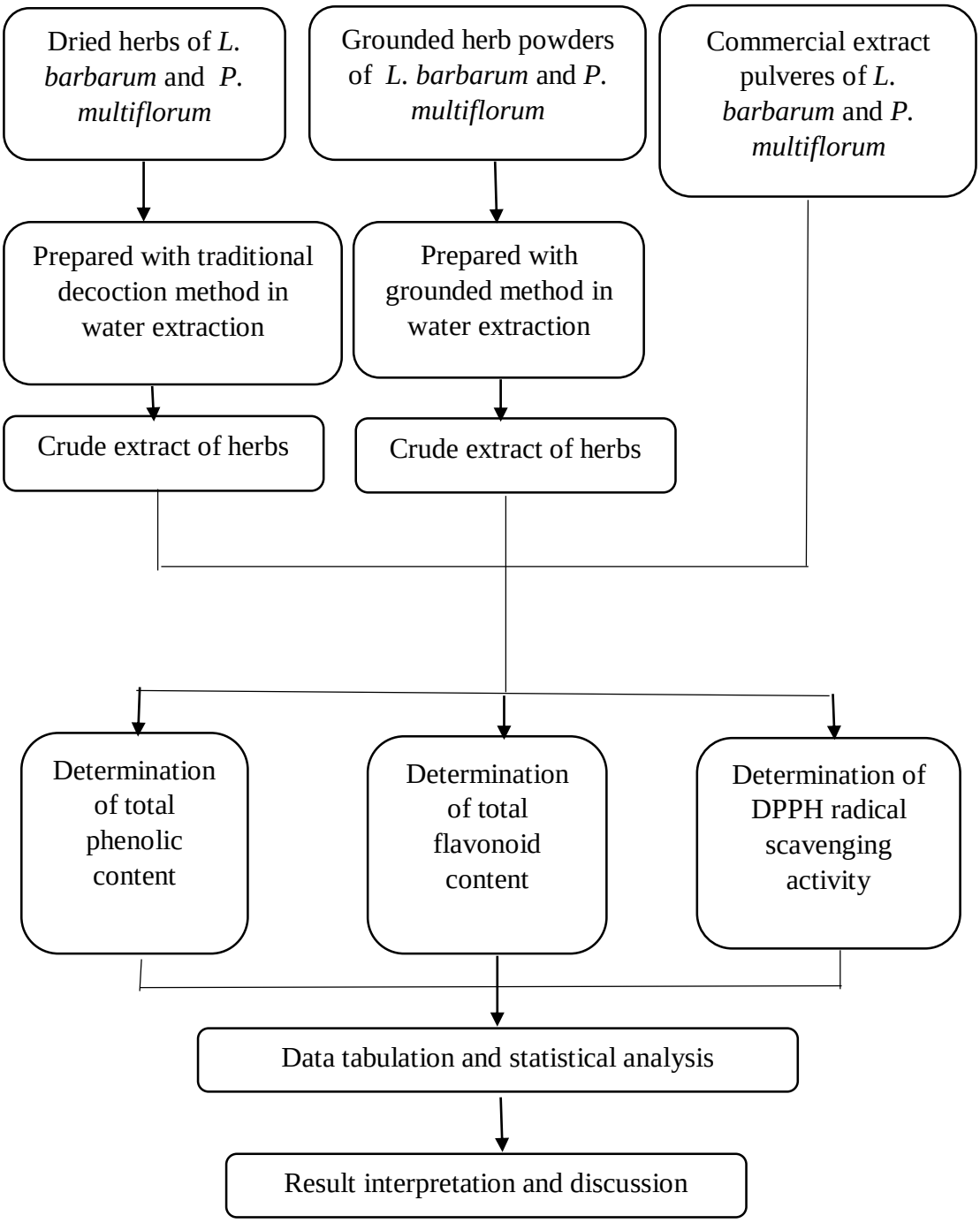

Figure 3.1: Overview of the methodology in this research.

3.5 Preparation of Crude Extract of Herbs

3.5.1 Decoction Method

Fifteen grams of dried herbs of *L. barbarum* were washed with running tap water and mixed with 150 mL distilled water in a conical flask. The conical flask was covered and wrapped with aluminium foil which acts as a light barrier for the herb samples. Then, the conical flask was boiled for 10 min and simmered for 110 min based on the traditional herbal decoction preparation method (Castleman, 2017).

After that, the sample solution was divided and poured into six centrifuge tubes with 20 ml, respectively, and centrifuged at 1,699 x *g* for 3 min (Li, et al., 2007). Next, the supernatant fluid was filtered with filter paper and cotton wool. The filtrate was then transferred into a rotary evaporator and was concentrated at 60 ℃. After evaporation, concentrated extract was transferred into a petri dish and dried in drying oven at 60 ℃ until constant weight was gained. The crude extract was then stored at -20 ℃ for further analysis. Same procedures were used for another sample, the dried herbs of *P. multiflorum*.

3.5.2 Grounded Method

Dried herbs of *L. barbarum* were grounded into powder form. Fifteen grams of the *L. barbarum* powder was measured using electrical balance and was mixed with 150 ml distilled water in a conical flask. To prevent leaking and light exposure of sample, the conical flask was wrapped with petrifilm and aluminium

foil. Next, the conical flask was placed into the shaking incubator and homogenized for 2 h at 64 x *g*. After that, the sample solution was divided and poured into six centrifuge tubes with 25 ml respectively, and then, centrifuged at 1,699 x *g* for 3 min (Li, et al., 2007). Then, the supernatant fluid was filtered with filter paper and cotton wool. The filtrate was then transferred into a rotary evaporator and concentrated at 60 ℃. After evaporation, concentrated extract was transferred into a petri dish and dried in drying oven at 60 ℃ until constant weight was reached. The crude extract was then stored at -20 ℃ for further analysis. Same procedures were used for the *P. multiflorum*.

3.6 Determination of Extraction Yield

The extraction yield of each crude extract was determined by using the Formula 3.1.

Formula 3.1:

$$\text{Yield of extract (\%)} = \frac{\text{Dry weight of crude extract obtained (g)}}{\text{Dry weight of herb used for extraction (g)}} \times 100$$

3.7 Antioxidant Assays

3.7.1 Preparation of Test Samples for Total Phenolic and Total Flavonoid Contents

Five milligrams of crude extract or extract pulveres was dissolved in 5 mL of distilled water in order to produce 1 mg/mL concentration solution for subsequent analyses. The sample solution was then mixed by using a vortex and covered with aluminium foil to prevent light exposure.

3.7.2 Determination of Total Phenolic Content

Total phenolic content (TPC) in each crude extract and commercial extract pulveres were determined using Folin-Ciocalteu (FC) Reagent Test as previously described by Waterhouse (2001) with slight modification.

3.7.2.1 Preparation of Sodium Carbonate Solution, Na_2CO_3

Sodium carbonate solution (20%; w/v) was prepared by dissolving 20 g of sodium carbonate powder with 100 mL distilled water in a 100 ml volumetric flask and kept at room temperature for further usage.

3.7.2.2 Preparation of Stock and Standard Solution of Gallic Acid

Gallic acid was selected as a reference standard for this assay. Stock solution of gallic acid with a concentration of 500 mg/L was prepared by mixing 0.005 g gallic acid powder with 10 ml of distilled water. The solution was properly

mixed and wrapped with aluminium foil as it is light sensitive. The stock solution was further diluted to 100 mg/L by adding 6 mL of 500 mg/L into 24 mL of distilled water. Next, several standard solutions with different concentrations (80, 60, 40, and 20 mg/L) were obtained from the 100 mg/L solution by performing serial dilutions using distilled water.

3.7.2.3 Folin-Ciocalteu Reagent Test

To start the Folin-Ciocalteu (FC) reagent test, 0.1 mL of FC reagent and 0.8 ml of distilled water was mixed with 0.2 mL of the prepared sample solution (1 mg/mL), then incubated for 3 min at room temperature. After that, the mixture was then added with 0.3 mL of 20% (w/v) sodium carbonate solution and incubated for 120 min at room temperature. After the colour development stage, the mixture was sent to vortex to ensure properly mixing before measuring the absorbance. Then, 200 µL of each mixture was transferred into a 96-well plate and measured at a wavelength of 765 nm. For the blank control, 0.2 mL of distilled water was used as a replacement of the sample solution. The same procedures were also applied to the gallic acid standard solutions. Absorbance values against concentration of gallic acid standard solutions were used to generate a standard calibration curve. Next, the total phenolic content of each extract was calculated based on the standard calibration curve and expressed as milligram gallic acid equivalent (GAE) per gram of dry weight (DW) of extract (mg GAE/g DW). All determinants were performed in triplicate and each test was repeated twice.

3.7.3 Determination of Total Flavonoid Content

Total flavonoid content (TFC) in each crude extract and commercial extract pulveres were determined using aluminium chloride colorimetric method as described previously by Pal, et al., (2014) with some modification.

3.7.3.1 Preparation of Sodium Nitrate Solution, $NaNO_3$

Sodium nitrate solution (5%; w/v) was prepared by mixing 5 g of sodium nitrate powder with 100 mL volume of distilled water in a 100 mL volumetric flask. This solution was properly mixed to ensure all powder was fully dissolved in water and kept at room temperature for further usage.

3.7.3.2 Preparation of Sodium Hydroxide Solution, NaOH

Sodium hydroxide solution (10%; w/v) was prepared by mixing 10 g of sodium hydroxide powder with 100 mL of distilled water in a 100 mL volumetric flask. This solution was properly mixed to ensure all powder was fully dissolved in water and kept at room temperature for further usage.

3.7.3.3 Preparation of Aluminium Chloride Solution, $AlCl_3$

Aluminium chloride solution (10%; w/v) was prepared by mixing 10 g of aluminium chloride powder with 100 mL methanol in a 100 mL volumetric flask. This solution was properly mixed to ensure all powder was completely dissolved and kept at room temperature for further usage.

3.7.3.4 Preparation of Stock and Standard Solution of Quercetin

Quercetin was selected as a reference standard for this assay. Quercetin stock solution with a concentration of 500 mg/L was prepared by mixing 0.005 g of quercetin powder with 10 mL of distilled water. This solution was properly mixed and wrapped with aluminium foil as it is light sensitive. The stock solution was further diluted to 100 mg/L by adding 6 mL of 500 mg/L into 24 mL of distilled water. Next, several standard solutions with different concentrations (80, 60, 40, and 20 mg/L) were obtained and prepared from the 100 mg/L solution with distilled water by performing serial dilution.

3.7.3.5 Aluminium Chloride Colourimetric Assay

To start the aluminium chloride colourimetric assay, 0.2 mL of the prepared sample solution (1mg/mL) was added to 0.15 mL of 5% (w/v) sodium nitrate solution and then incubated for 6 min at room temperature. After that, 0.15 mL of 10% (w/v) aluminium chloride solution was added to the mixture and the mixture was incubated for another 6 min at room temperature. Next, 0.8 mL of 10% (w/v) sodium hydroxide solution was added to the mixture and was allowed to stand for 15 min. Then, the mixture was sent to vortex before measuring the absorbance. A volume of 200 µL of each mixture was transferred into a 96-well plate and the absorbance of the mixtures was measured at 510 nm. For the blank control, 0.2 mL of distilled water was used as a replacement of the sample solution. The same procedures were also applied to the standard solutions of quercetin. Absorbance values against concentration of quercetin standard

solutions were used to generate a standard calibration curve. The total flavonoid content of each extract was calculated based on the standard calibration curve and was expressed as milligram quercetin equivalent (QE) per gram of dry weight (DW) of extract (mg QE/g DW). All determinants were carried out in triplicate and each test was repeated twice.

3.7.4 Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of each crude extract and commercial extract pulveres were assessed using the DPPH assay as previously described by Chai and Wong, (2012) with slight modification.

3.7.4.1 Preparation of Test Samples

Five milligrams of crude extract or extract pulveres was dissolved in 5 mL of distilled water to produce a stock solution with 1 mg/mL concentration. The sample solution was then mixed by a vortex and covered with aluminium foil to prevent light exposure. Then, several sample solutions with different concentrations that range from 10 μg/mL to 500 μg/mL were prepared by carried out serial dilutions from the stock solution. All prepared sample solutions were mixed by using a vortex and covered with aluminium foil to prevent light exposure.

3.7.4.2 Preparation of DPPH Solution

DPPH solution was freshly prepared on the day before the assay was performed. DPPH powder with amount of 1.97 mg was mixed with 50 mL of methanol to produce a 0.1 mM DPPH solution. This solution was then covered with aluminium foil to prevent light exposure and stored in refrigerator at 4℃ until usage.

3.7.4.3 Preparation of Ascorbic Acid Solution

Ascorbic acid was used as a reference standard in this assay. One milligram of ascorbic acid powder was mixed with 10 mL of methanol to produce a stock solution with 100 μg/mL concentration and the stock solution was further diluted to 50, 25, 20, 15, 10, 5 μg/mL by performing serial dilution. All ascorbic acid solution with different concentrations were properly mixed, covered and wrapped with aluminium foil, and then kept at room temperature for future usage.

3.7.4.4 DPPH Radical Scavenging Activity Assay

To begin the DPPH assay, 0.5 mL of the prepared sample solution was mixed with 0.5 mL of the (0.1 mM) DPPH solution using a vortex. The mixture was then incubated under dark environment for 30 min at room temperature. After incubation, a volume of 200 μL of each mixture was transferred into a 96-well plate and the absorbance of the mixtures was measured at 517 nm. Ascorbic acid solution with different concentrations were used as references and all of the steps mentioned above were repeated. A mixture that contained same amount of

methanol and DPPH solution was used as negative control meanwhile methanol was used as blank. All determinants were carried out in triplicate and each test was repeated twice.

The DPPH radical scavenging activity percentage of each extracts and ascorbic acid were calculated based on the Formula 3.2. Then, the results of this assay were expressed as EC_{50}, the concentration of sample needed to achieve 50 % of radical scavenging activity, through linear regression analysis.

Formula 3.2:

DPPH free radical scavenging activity (%) = [1- (As/ Ac)] x 100 %

where As = Absorbance of sample and Ac = Absorbance of negative control

3.8 Statistical Analysis

All results were tabulated and presented as mean ± standard deviation (SD). All standard curve calibration and calculation of TPC and TFC of each test sample, as well as linear regression analyses of EC_{50} were performed using Microsoft Excel 2013.

Then, the data were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's test for post-hoc analysis to detect significant differences between means of treatments at $P < 0.05$. Besides, independent samples T-test was employed to determine the difference between two herb samples of a particular preparation method. In DPPH assay, independent samples T-test was also applied to assess the difference between test samples and ascorbic acid. Next, Pearson correlation was used to determine the relationships between TPC, TFC, and EC_{50} at significance level at 0.01. These statistical analyses were generated using SPSS statistical software (version 20; SPSS Inc., Chicago, IL).

CHAPTER 4

RESULTS

4.1 Extraction Yield

The extraction yield of each crude extract was summarised in Table 4.1. As shown in the table, a significant ($P < 0.05$) higher yield was obtained from *L. barbarum* compared to that of *P. multiflorum*, regardless the preparation methods used. The crude extract of *L. barbarum* prepared by grounded method showed the highest percentage of yield followed by the crude extract of *L. barbarum* prepared by decoction method, crude extract of *P. multiflorum* prepared by grounded method and crude extract of *P. multiflorum* prepared by decoction method.

Table 4.1: Extract yields of herbs with different preparation methods.

Percentage Yield of Extracts (%)		
Type of Herbs	**Preparation Methods**	
	Decoction	**Grounded**
L. barbarum	11.06^{aA}	11.73^{aA}
P. multiflorum	8.87^{bB}	10.40^{bB}

Values in the same row followed by different superscript small capital letters are significantly different as determined by ANOVA test ($P < 0.05$). Values in the same column followed by different superscript big capital letters are significantly different as determined by ANOVA test ($P < 0.05$).

4.2 Antioxidant Assays

4.2.1 Total Phenolic Content

A standard curve was generated using absorbance values of gallic acid against different concentrations, as shown in Figure 4.1. The total phenolic content (TPC) of each crude extract and extract pulveres of herbs were obtained based on calculation from the standard calibration curve and recorded in Table 4.2. Among the preparation methods used, an increase in the TPC was observed for the crude extract of *P. multiflorum* extracted using decoction method compared to that of grounded and commercial extract pulveres. On the other hand, among the preparation methods used for *L. barbarum*, the crude extract of *L. barbarum* extracted using grounded method showed the highest TPC compared to that of decoction and commercial extract pulveres. Overall, the *P. multiflorum* extracts showed a higher TPC compared to *L. barbarum* extracts within the same preparation methods except for the grounded method. The TPC for all the extracts were significantly different with one another ($P > 0.05$).

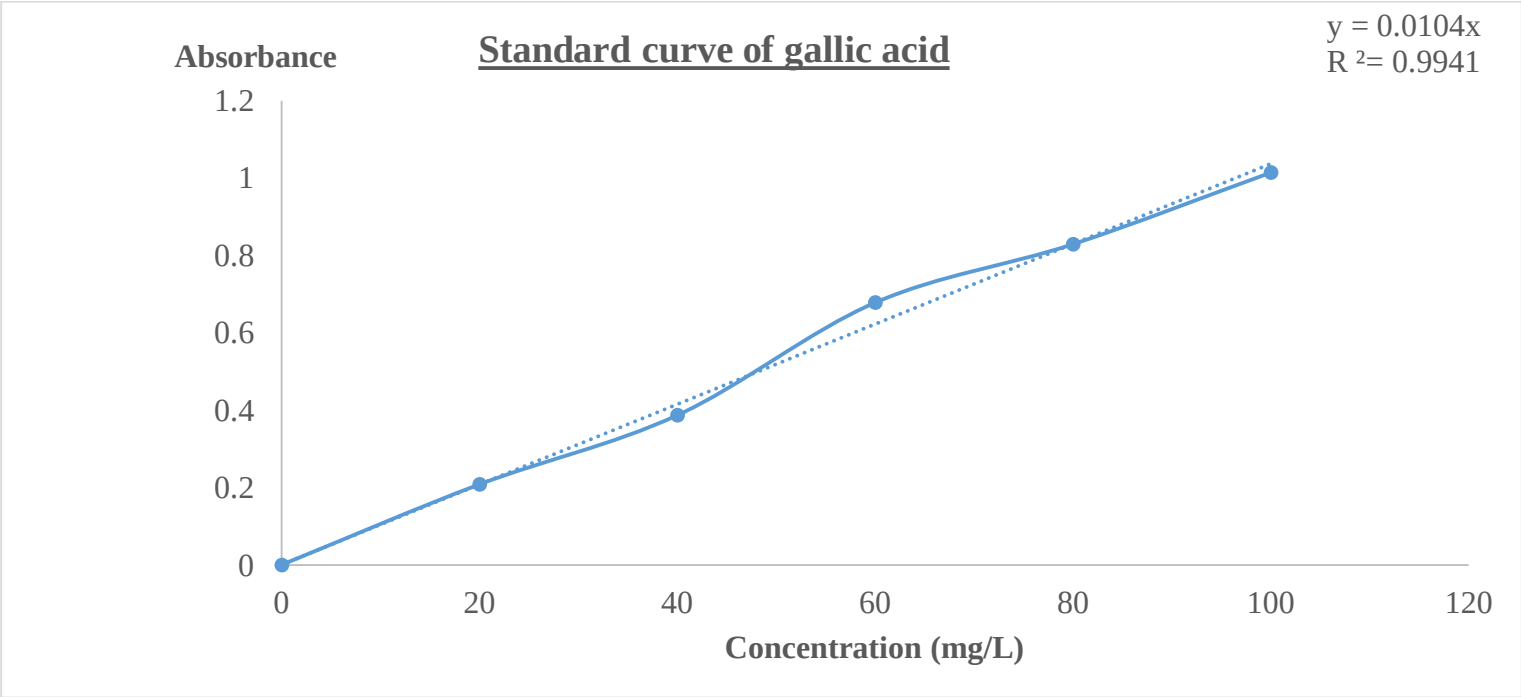

Figure 4.1: Standard curve of absorbance against concentration of gallic acid.

Table 4.2: Total phenolic content of each crude extract and extract pulveres of herbs (mg GAE/g DW).

Total phenolic content (mg GAE/g DW)			
Type of Herb	**Preparation methods**		
	Decoction	**Grounded**	**Commercial Extract Pulveres**
L. barbarum	24.23 ± 1.14^{aA}	57.10 ± 1.72^{bA}	28.64 ± 1.82^{cA}
P. multiflorum	70.21 ± 5.18^{aB}	45.59 ± 2.98^{bB}	55.09 ± 3.29^{cB}

Data are presented as mean ± standard deviation (SD) (n=6). Values in the same row followed by different superscript small capital letters are significantly different as determined by Tukey test ($P < 0.05$). Values in the same column followed by different superscript big capital letters are significantly different as determined by independent samples T-test ($P < 0.05$).

4.2.2 Total Flavonoid Content

A standard curve was generated by using absorbance values of quercetin at different concentrations, as shown in Figure 4.2. The total flavonoid content (TFC) of each crude extract and extract pulveres of herbs were obtained based on calculation from the standard calibration curve and recorded in Table 4.3. The results showed a similar pattern to the results of TPC. The TFC for all the extracts were significantly different with one another ($P > 0.05$). Among the preparation methods used, a decrease in the TFC was observed in the crude extract of *P. multiflorum* extracted using grounded method compared to that of decoction and commercial extract pulveres. It (38.30 mg QE/g DW) had only 22.72% of the TFC recorded by the crude extract of *P. multiflorum* extracted using decoction method (168.57 mg QE/g DW). In contrast, among the preparation methods used for *L. barbarum*, the crude extract of *L. barbarum* extracted using decoction method showed the lowest TFC compared to that of grounded method and commercial extract pulveres. It (26.25 mg QE/g DW) had only 18.49 % of the TFC recorded by the crude extract of *L. barbarum* extracted using grounded method (152.80 mg QE/g DW). Overall, the *P. multiflorum* extracts showed a higher TFC compared to *L. barbarum* extracts within the same preparation methods except for the grounded method

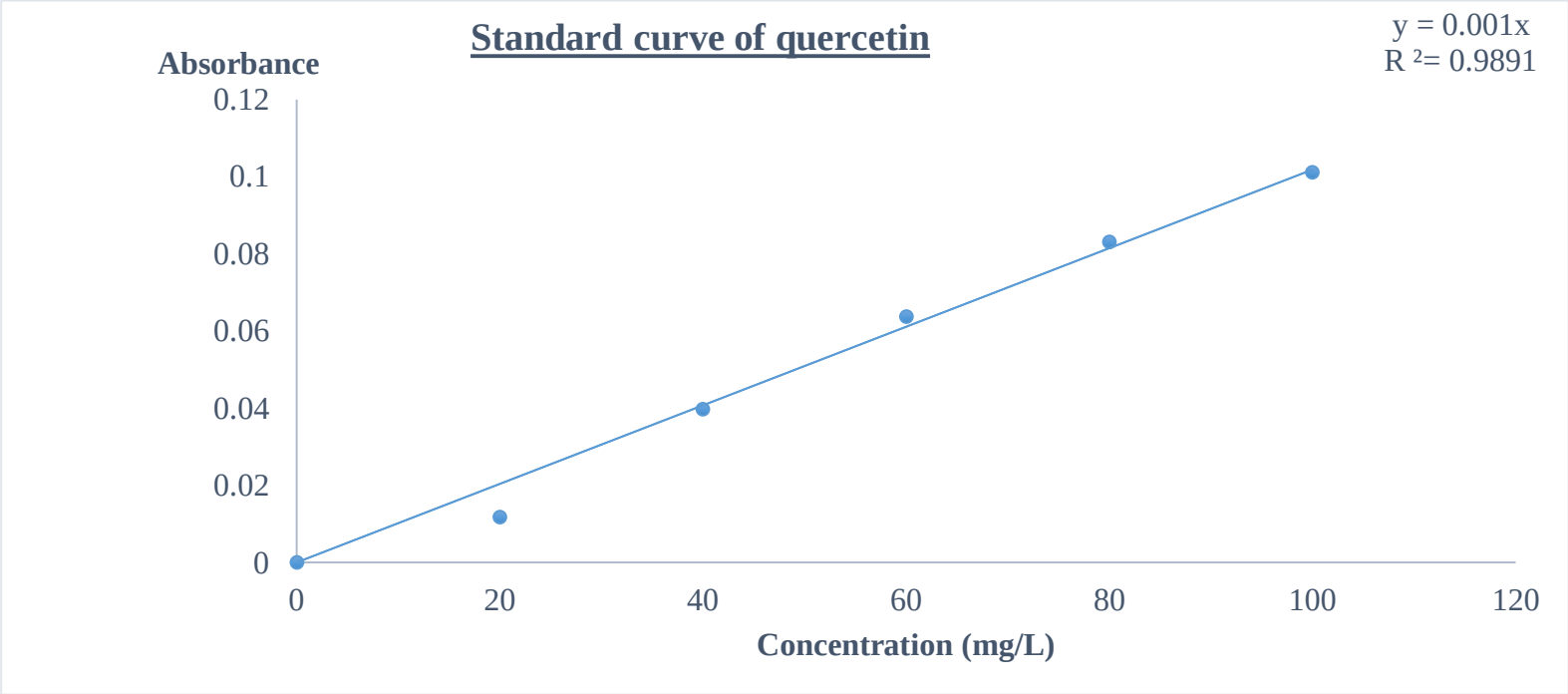

Figure 4.2: Standard curve of absorbance against concentration of quercetin.

Table 4.3: Total flavonoid content of each crude extract and extract pulveres of herbs expressed as (mg QE/g DW).

Total Flavonoid Content (mg QE/g DW)			
Type of Herbs	**Preparation methods**		
	Decoction	**Grounded**	**Commercial Extract Pulveres**
L. barbarum	26.25 ± 3.26^{Aa}	152.80 ± 6.47^{Ba}	37.56 ± 4.74^{Ca}
P. multiflorum	168.57 ± 6.36^{aB}	38.30 ± 3.85^{bB}	95.95 ± 3.95^{cB}

Data are presented as mean ± standard deviation (SD) (n=6). Values in the same row followed by different superscript small capital letters are significantly different as determined by Tukey test ($P < 0.05$). Values in the same column followed by different superscript big capital letters are significantly different as determined by independent samples T-test ($P < 0.05$).

4.2.3 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of ascorbic acid and sample solutions with different concentrations were ranged from 0 μg/ml to 500 μg/mL as shown in Figure 4.3. Based on the graph in Figure 4.3, a dose-dependent relationship was observed whereby DPPH radical scavenging activity of all samples increased in response to the increasing concentrations. The concentration of each sample needed to achieve 50% of radical scavenging activity, is known as EC_{50}, was obtained through linear regression analysis and presented in Table 4.4. As shown in Table 4.4, the descending order based on the value of EC_{50} was as follows: commercial extract pulveres of *L. barbarum* > *L. barbarum* extract prepared by decoction method > *P. multiflorum* extract prepared by grounded method > commercial extract pulveres of *P. multiflorum* > *L. barbarum* extract prepared by grounded method > *P. multiflorum* extract prepared by decoction method > ascorbic acid.

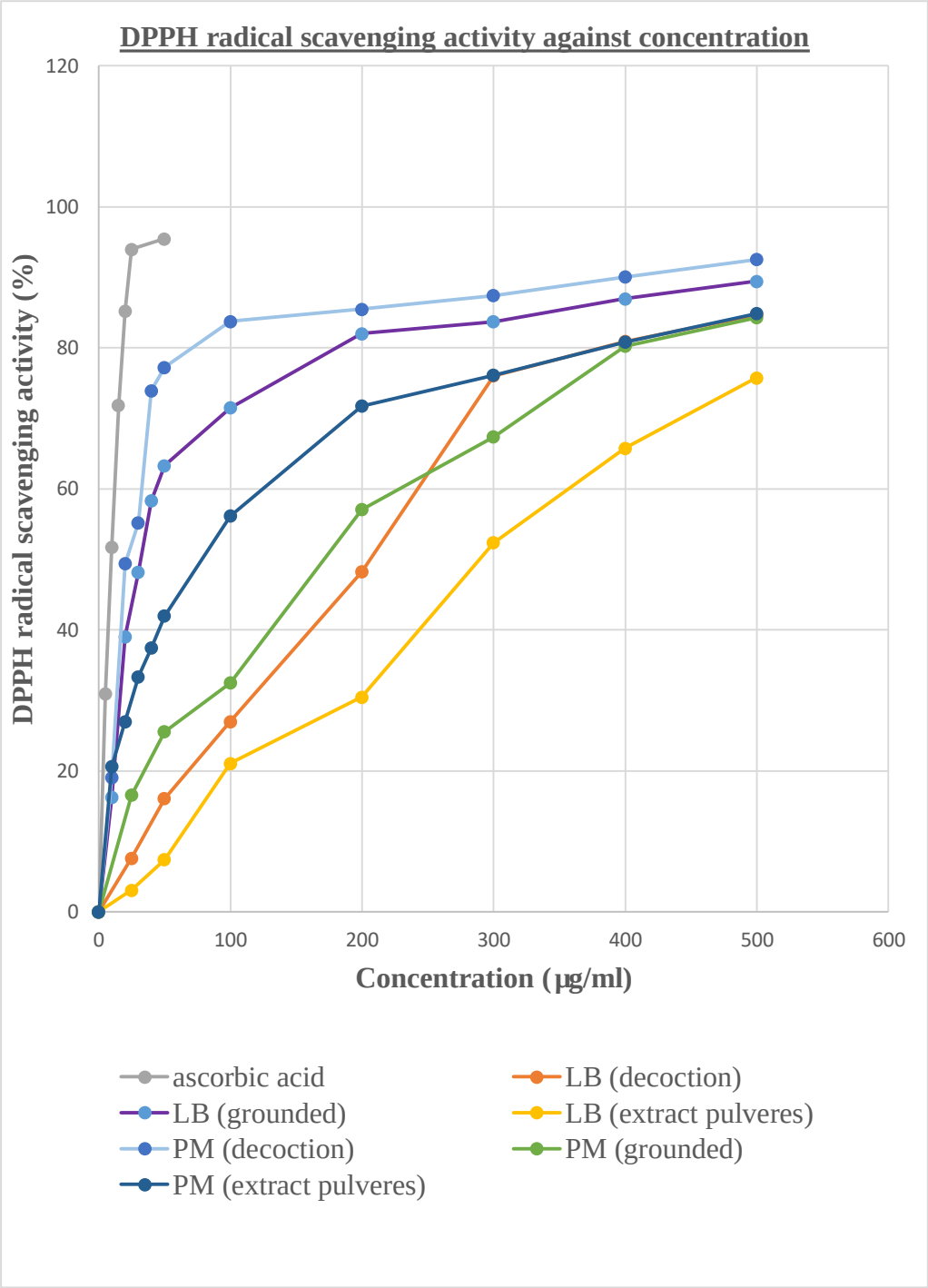

Figure 4.3: DPPH radical scavenging activity against concentration of all samples. The tested samples were ascorbic acid, four different crude extract of *L. barbarum* (LB) and *P. multiflorum* (PM) prepared by decoction and grounded methods, and two commercial extract pulveres of these two herbs. Data are presented as mean ± standard deviation (n=6).

Table 4.4: The EC_{50} values of test samples based on DPPH radical scavenging activity.

	EC_{50} (μg/mL)		
Ascorbic acid	9.29 ± 0.04		
Type of Herbs	**Preparation Methods**		
	Decoction	**Grounded**	**Commercial Extract Pulveres**
L. barbarum	$215.47 \pm 2.51^{aA*}$	$34.19 \pm 0.32^{bA*}$	$297.93 \pm 3.80^{cA*}$
P. multiflorum	$24.22 \pm 0.14^{aB*}$	$187.65 \pm 2.86^{bB*}$	$68.34 \pm 0.35^{cB*}$

Data are presented as mean ± standard deviation (SD) (n=6). Values in the same row followed by different superscript small capital letters are significantly different as determined by Tukey test ($P < 0.05$). Values in the same column followed by different superscript big capital letters are significantly different as determined by independent samples T-test ($P < 0.05$). Superscript * indicates mean values of herb's extract which are significantly different ($P < 0.05$) from that of the mean value of ascorbic acid, as determined by using independent samples T-test.

4.3 Correlation Analysis

The correlations between analysed parameters were determined by using the Pearson correlation in SPSS software (version 20). The correlation coefficient (r) between different parameters was presented in Table 4.5. From the table, all values presented between different test parameters were statistically significant ($P < 0.01$), which indicated there are relationships between these parameters. Besides a strong positive correlation between TPC and TFC ($r = 0.884$), strong negative correlations between TPC and EC_{50} ($r = -0.896$), and between TFC and EC_{50} ($r = -0.904$), respectively were also presented in Table 4.5. A positive correlation showed that an increase in TPC when TFC was increased. Negative correlations also revealed that EC_{50} decreased when TPC and TFC were increased.

Table 4.5: Correlations analysis between total phenolic content (TPC), total flavonoid content (TFC), and EC_{50}.

Parameters	Correlation coefficient (r)		
	TPC	**TFC**	**EC_{50}**
TPC	1	0.884**	-0.896**
TFC	0.884**	1	-0.904**
EC_{50}	-0.896**	-0.904**	1

** indicates values presented are statistically significant at $P < 0.01$.

CHAPTER 5

DISCUSSION

5.1 Percentage Yield of Extraction

Yield of extraction is defined as the amount of extract recovered in mass as compared with the initial amount of original material. It is usually expressed in percentage (Murugan and Parimelazhagan, 2014). It is also used to indicate solvent's efficiency in extracting certain components such as phytochemicals from the original material (Murugan and Parimelazhagan, 2014). Suitable extraction technique is crucial to obtain a high yield of extract with desired phytochemicals for further analysis. Different extraction methods and solvent systems are available to extract the phytochemicals of the TCM herbs. For instance, various extraction methods such as rotary evaporation, heating under reflux, and soxhlet extraction can be employed to extract the plant samples as well as TCM herbs (Sasidharan, et al., 2010; Seo, et al., 2014). The polarity of extraction solvent has also been found to have an effect on polyphenol content and antioxidant activity of medicinal plants (Hayouni, et al., 2007).

In this study, the focus was on the effect of different preparation methods on the herbs' antioxidant properties prior to consumption. Thus, the herbs were selected on the basis of traditional uses and were prepared as described by the traditional Chinese medicine practitioner (Fabricant and Farnsworth, 2001). It has been revealed that antioxidant compounds in *L. barbarum* fruits and *P. multiflorum*

roots are mostly hydrophilic compounds and readily dissolved in water (Chan, et al., 2010; Yang, et al., 2015). Thus, water extraction was used in this study and the crude extract was obtained using rotary evaporation technique. Proper operations must be taken during the extraction process to ensure the potential phytochemicals are not lost or destroyed (Sasidharan, et al., 2010).

Based on the results, the percentage yield of all extraction was ranged from 8.87% to 11.73%. Yang, et al. (2015) have previously reported that the extraction yield of crude *L. barbarum* polysaccharides using hot water extraction was 7.63% while ultrasound-enhanced subcritical water extraction was able to obtain 14.1% of yield.

There was no significant difference ($P > 0.05$) observed between the crude extracts of a particular herb prepared by two different preparation methods as shown in Table 4.1. However, the crude extract of a particular herb prepared by grounded method showed a slightly higher percentage compared to that of decoction method. This was due to the particle size of herbs. Herbs were ground into powdered form in grounded method and this eventually enhanced the percentage of extraction yield due to increaased surface area to volume ratio. The smaller the size of particle, the higher the surface area to volume ratio that come into contact with the solvent (Sasidharan, et al., 2011). This helps in formation of non-covalent bonds with functional groups of phytochemicals and allows easier extraction of the phytochemicals (Azmir, et al., 2013).

Apart from that, the crude extracts of *L. barbarum* also showed a significantly ($P < 0.001$) higher percentage yield compared to the crude extracts of *P. multiflorum* within the same preparation method. This was due to different chemical properties of the herbs. It has been reported that *L. barbarum* fruit contained hydrophilic compounds that are weighty such as *L. barbarum* polysaccharides (Yang, et al., 2015). Besides, the size of *L. barbarum* fruits used in this study was smaller and had a higher surface to volume ratio compared to the root slices of *P. multiflorum*. This may subsequently increase the efficiency and percentage yield of extraction.

5.2 Total Phenolic Content

Polyphenols or phenolic compounds of medicinal herbs are notable as primary antioxidant compounds for their free radical scavenging activity. Phenolic compounds composed of an aromatic benzene ring with substituted functional derivatives and hydroxyl groups that make them have the ability to scavenge free radicals, prevent lipid peroxidation, as well as chelate metal ions (Iqbal, Salim and Lim, 2015).

As shown in Table 4.2, the TPC in extracts of *L. barbarum* in this study was ranged from 24.23 ± 1.14 to 57.10 ± 1.72 mg GAE/g DW. This result was comparable to the study of Benchennouf, et al. (2016). The authors reported that the TPC of *L. barbarum* cultivated in Greece was ranged from 14.13 (water extract) to 109.72 (ethyl acetate extract) mg GAE/g DW. Another study conducted by Reis, et al. (2014) also documented that the TPC of *L. barbarum*

was 15.67 ± 1.23 mg GAE/g DW before fermentation. All the *L. barbarum* extracts that tested in the present study showed a slightly higher TPC compared to the water extracts of *L. barbarum* in both studies of Benchennouf, et al. (2016) and Reis, et al. (2014), but showed a lower TPC compared to the TPC of *L. barbarum* that were extracted by other solvents. This was due to a higher polarity of solvents used to extract phenolic compounds compared to the water (Rao, Abdurrazak and Mohd, 2016).

In fact, the *L. barbarum* fruits have scopoletin and other coumarin derivative compounds as their major types of phenolic compounds (Cai, et al., 2004). Coumarin is a large class of plant secondary metabolites that widely distributed in various plant families. Coumarin and its derivatives such as scopoletin and marmesin are considered as phenylpropanoids and biosynthesized from shikimic acid-derived phenylpropane precursors (Molnar, et al., 2017). They are found not only to possess antioxidant properties but also documented to have anti-inflammatory and antimicrobial properties (Molnar, et al., 2017). Besides, scopoletin also has the antioxidant properties due to its superoxide anion scavenging activity (Shaw, et al., 2003).

Among the extracts of *L. barbarum*, the lowest TPC was observed in the crude extract prepared by decoction method. This was due to the loss of the phenolic compounds of *L. barbarum* during the high heat treatment using the decoction method. A similar observation was obtained by Sikora, et al. (2008), the authors found that aquathermal processes can lead to the loss of polyphenols into the water environment. The commercial extract pulveres, which usually produced

after several processing steps that involved heat treatment, had a significant lower phenolic content compared to the crude extract prepared by grounded method. The more the processing steps, the easier the loss of bioactive compounds of the herb (Sasidharan, et al., 2010). Therefore, commercial extract pulveres of *L .barbarum* cannot retain the phenolic compounds as many as the crude extract of *L. barabarum* prepared by grounded method.

Meanwhile, the TPC for the extracts of *P. multiflorum* in this study were ranged from 45.59 ± 2.98 to 70.21 ± 5.18 mg GAE/g DW. These results were comparable to the study of Quoc and Muoi (2016), the TPC of *P. multiflorum* Thunb root was reported as 44.37 ± 0.13 mg GAE/g DW using microwave-assisted extraction (MAE). A similar observation was also reported by Ho, et al., (2017), where a 53.87 mg GAE/g DW of TPC was obtained from root samples based on 5 g/L inoculum density. However, the TPC values obtained for the extracts of *P. multiflorum* in this study were lower compared to a previous study done by Lin, et al. (2010), whereby 179.0 ± 11.1 mg GAE /g DW of TPC was determined for water extract of *P.multiflorum* roots.

Main phenolic compounds found in *P.multiflorum* root are anthraquinones (emodin, chrysophanol, physcion, rhein), tannins, and stilbenes (resveratrol) (Cai, et al., 2004). Anthraquinones such as emodin, chrysophanol, physcion, rhein are secondary metabolites with an anthracene skeleton (Wink, 2015). Anthraquinones carry phenolic hydroxyl group and able to interfere with proteins (Wink, 2015). Besides, tannins are strong antioxidants whereas stilbenes, such as resveratrol that usually present in red wine, are well known for

their antioxidant, antibacterial, and antifungal activities (Wink, 2015). Hence, these phenolic compounds of *P. multiflorum* root are responsible for its antioxidant properties.

Among the extracts of *P. multiflorum*, the highest TPC was determined in the crude extract prepared by decoction method. This was in agreement with Xu, et al. 2007, who evaluated the effect of heat treatment process on phenolic compounds of food and found that the TPC was increased upon the heat treatment. This is because the polyphenol oxidase (PPO) enzyme is inactivated by heat treatment during decoction method, and thus inhibits polyphenolics degradation (Shaimaa, et al., 2016). After that, the commercial extract pulveres of *P. multiflorum* had significantly higher TPC compared to the crude extract prepared by grounded method due to heat treatment may be applied by the manufacturer to produce the extract pulveres. Furthermore, there may be oxidative degradation of polyphenols in grounded *P. multiflorum* due to the reaction between cytoplasmic PPO and phenolic compounds present in the vacuoles during grinding (Manach, et al., 2004). Therefore, crude extract of *P. multiflorum* prepared by grounded method showed the lowest TPC among the extract of *P. multiflorum*.

The TPC results among the extracts of *P. multiflorum* were not comparable to the TPC results of the extracts of *L. barbarum* due to the food matrix of herbs are different (Leitao, et al., 2011). The food matrix can induce the degradation of phenolic compounds or act as protective barrier towards heat effect (Freeman, Eggett and Parker, 2010). Comparing the type of herbs used in this study, *P.*

multiflorum showed a higher TPC values for each preparation method except for the crude extract prepared by grounded method. This was in agreement with Cai, et al. (2004), whereby the *P. multiflorum* roots had 4.18 g/100 g DW phenolic content in aqueous extract meanwhile the *L. barbarum* fruits had 0.70 g/100 g DW phenolic content in aqueous extract.

5.3 Total Flavonoid Content

Among polyphenols, flavonoids are vital and famous for their ability to help human body to fight against free radicals. Flavonoids are ubiquitous groups of polyphenolic compounds and abundantly found in medicinal plants (Iqbal, Salim and Lim, 2015). The ability of flavonoids to act as potent antioxidants are depend on their molecular structures, their hydroxyl group's position, and other features in its chemical structure (Iqbal, Salim and Lim, 2015).

Based on the results obtained, there were significantly different among all the extracts ($P > 0.05$). The TFC for the extracts of *L. barbarum* were ranged from 26.25 to 152.80 mg QE/g DW. This was comparable to the study of Oguz and Erdoga (2016), where the authors found that the TFC of *L. barbarum* fruits were ranged from 48.89 to 51.09 mg QE/g. However, a lower TFC in *L. barbarum* was reported (1.56 mg QE/ g extract) in the study of Le, Chiu and Ng (2007). Istrati, et al. (2013) also found that the TFC of *L. barbarum* fruit was 53.06 ± 1.23 mg QE/100 g fruit material. The authors also revealed that the TFC of several *L. barbarum* fruits were ranged from 2.67 to 3.16 mg catechin equivalents (CAE)/g sample. There are few possible factors that may cause a

variation in the results such as the procedures employed in the processing of herbs to form crude extracts, the evaluation methods, and also the origin or location of the plantation of herbs (Abrahim, Kanthimathi and Abdul-Aziz, 2012).

Quercetin-3-O-rutinoside and quercetin-rhamno-di-hexoside were detected as predominat flavonoids in *L. barbarum* fruit by Zhang, et al. (2016). Moreover, Benchennouf, et al. (2016) also reported that the flavonoids such as quercetin 3-*O*-hexose-*O*-hexose-*O*-rhamnose and quercetin 3-*O*-hexose coumaric ester were found in *L. barbarum* fruits. Other identified flavonoids found in *L. barbarum* fruit were rutin, catechin and epicatechin (Donno, et al., 2015).

Among the extracts of *L. barbarum*, the crude extract prepared by decoction method showed the lowest TFC. This might due to degradation of flavonoids occurs during the boiling step in the decoction method. This was in agreement with the study of Ranilla, Genovese and Lajolo, (2009), the authors found that boiling was able to induce the loss of quercetin and kaempferol up to 90 %. Besides, there may be leaching of quercetin and other flavonoids from *L. barbarum* fruit into the boiling water (Sharma, et al., 2015). The commercial extract pulveres showed a significant ($P < 0.05$) lower TFC compared to the crude extract prepared by grounded due to heat treatment may be applied during the manufacturing process. In fact, flavonoids contain C–glycoside bonds and usually exist as dimers and oligomers in food, but heating or boiling process will lead to the hydrolysis of C–glycosides bonds and results in the formation of

monomers (Manach, et al., 2004; Sharma, et al., 2015). Therefore, commercial extract pulveres cannot retain flavonoids as many as the crude extract of *L. barabarum* prepared by grounded method.

On the other hand, the TFC in the extracts of *P. multiflorum* either grounded or decoction method were ranged from 38.30 to 168.57 mg QE/g DW, which were at least 5 times higher than the result determined by Lin, et al. (2010). The authors reported that the TFC of the root extract of *P. multiflorum* was 7.3 ± 0.1 mg QE/g extract. Moreover, Thiruvengadam, et al. (2016) also reported that the TFC in several cell suspension cultures of *P. multiflorum* were ranged from 0.07 to 0.16 mg QE/g extract. These different results obtained may be caused by different processing steps involved to obtain the crude extracts from the herbs and the analysis methods used (Abrahim, Kanthimathi and Abdul-Aziz, 2012).

Examples of flavonoids found in *P. multiflorum* root are tricin, rutin, quercetin, kaempferol, and quercetin-3-O-arabinoside (Lin, et al., 2015). Besides, Chen, et al. (2012) also found a novel flavonostilbene glycoside, which was identified as polygonflavanol A, from the root of *P. multiflorum*. All of the identified flavonoids are found to exhibit antioxidant properties and free radical scavenging activities (Lin, et al., 2015).

Among the extracts of *P. multiflorum*, the crude extract prepared by decoction method showed the highest TFC. This is because the heat treatment (boiling) during decoction method is able to disrupt the cell walls of *P. multiflorum* root, liberates soluble phenolic compounds such as flavonoids from the insoluble ester

bonds (Ornelas-Paz et al., 2010). Hence, more flavonoids were released and detected in the crude extract of *P. multiflorum* prepared by decoction method compared to others. Furthermore, the commercial extract pulveres of *P. multiflorum* had a significant higher TFC ($P < 0.05$) compared to the crude extract prepared by grounded method. This might due to the application of heat treatment by the manufacturer during the production of the extract pulveres which enable the release of more flavonoids from *P. multiflorum*. Therefore, the crude extract of *P. multiflorum* prepared by grounded method, which did not involve any heat treatment, showed the lowest TFC among the extract of *P. multiflorum*.

The different types of food matrix of herbs have caused the result of *P. multiflorum* extracts dissimilar to the result of *L. barbarum* extracts whereby the highest flavonoids content obtained by decoction method and grounded method (Leitao, et al., 2011). Different structure of particular flavonoids present in the herbs also will cause the different results when they prepared using different methods (Sharma, et al., 2015). While in comparison to *L. barbarum*, *P. multiflorum* showed a higher TFC values for all preparation methods except for the crude extract prepared by grounded method. This was similar with the results obtained in TPC values. Thus, a low amount of phenolic compounds in particular extract also indicates a low amount of flavonoids.

5.4 DPPH Radical Scavenging Activity

Antioxidant compounds of herb extracts are found to have the ability to reduce the stable DPPH free radical from purple colour to the yellow coloured diphenylpicrylhydrazine (Onoja, et al., 2014). In DPPH assay, the degree of discoloration of the DPPH solution indicates the antioxidant activity of the herb extracts based on the herb extracts' hydrogen donating ability (Onoja, et al., 2014).

According to Lu, Khoo and Wiart, (2014), EC_{50} is a typical parameter that usually employed to express the antioxidant capacity of medicinal plants or herbs. EC_{50} value, represents the concentration of herb extracts in order to scavenge 50% of the DPPH free radicals. It is inversely proportional to the percentage of DPPH radical scavenging activity, in which compound that showed a higher percentage of scavenging activity, exhibited a lower EC_{50}, and are said to possess a greater antioxidant activity (Lu, Khoo and Wiart, 2014).

Based on the results, ascorbic acid showed the highest antioxidant activity with significant lowest EC_{50} value ($P < 0.05$) compared to all of the herb extracts. This is because ascorbic acid is a pure compound without any impurities and other mixtures of element that might interfere with its scavenging activity (Somawathi, et al., 2015). Therefore, herb extracts, which are mixture of compounds, showed less effectiveness as compared to ascorbic acid in term of scavenging activity and antioxidant activity.

Besides, a dose dependent scavenging activity was showed by all extracts including ascorbic acid. In a dose dependent scavenging activity, the percentage of radical scavenging activity will be extended with the concentration of the extracts (Pavithra and Vadivukkarasi, 2015). This phenomenon occurrs due to a higher concentration of extract might consist of a higher amount of antioxidant compounds and thus exhibit a higher scavenging activity (Pavithra and Vadivukkarasi, 2015). Meanwhile, a low concentration of extract has difficulty in scavenging the DPPH free radical due to limited amount of antioxidant compounds present (Pavithra and Vadivukkarasi, 2015).

On the other hand, the EC_{50} values of *L. barbarum* extracts were ranged from 34.19 to 297.93 μg/mL. In contrast, Benchennouf, et al. (2016) found that the EC_{50} of *L. barbarum* fruit extracted by several solvents, were ranged from 4.73 mg/mL (ethyl acetate extract) to 42.76 mg/mL (water extract). Besides, a study of Ruffo, et al. (2017) found that the DPPH inhibition activity of Chinese *L. barbarum* fruit was 35% at 0.15 mg/mL concentration. Another study conducted by Zhang, et al. (2016) also documented that the DPPH radical scavenging activity of several genotypes of *L. barbarum* fruit were ranged from 35.88 to 85.46 μM trolox equivalents /g fruit weight.

Among the extracts of *L. barbarum*, crude extract of *L. barbarum* prepared by grounded method showed the lowest EC_{50}. This also indicates the highest DPPH radical scavenging activity among the extracts. It has been suggested that large amount of phenolic compounds and flavonoids can contribute to a high free radical scavenging activity (Chai and Wong, 2012). This was in tandem with a

high TPC and TFC present in the crude extract of *L. barbarum* prepared by grounded method. On the contrary, the commercial extract pulveres showed the highest EC_{50}, which indicates it had the lowest free radical scavenging activity although the TPC and TFC were higher than the crude extract prepared by decoction method. This is because the determined amount of total phenolic compounds do not quantify or represent all the number of antioxidant compounds present in *L. barbarum* (Yi, Liu and Dong, 2013). The antioxidant compounds present in *L. barbarum* extracts also consist of *L. barbarum* polysaccharides (LBP) that are hydrophilic besides phenolic compounds (Yi, Liu and Dong, 2013).

On the other hand, the extracts of *P. multiflorum* exhibited EC_{50} that were ranged from 24.22 to 187.65 μg/mL as shown in Table 4.4. It is hard to compare the present result to other study because there is limited number of study in determining the DPPH radical scavenging activity of *P. multiflorum* extract and most of them expressed the results in different ways instead of EC_{50}. For instance, Ho, et al. (2017) determined the percentage of inhibiting the DPPH activity in several adventitious root culture of *P. multiflorum* were ranged from 85 to 90%.

Among extracts of *P. multiflorum*, the crude extract prepared by decoction method showed the lowest EC_{50}, which means the greatest DPPH radical scavenging activity compared to the crude extract prepared by grounded method. This was contributed by a higher TPC and TFC present in the crude extract prepared by decoction method. It has been documented that the main

antioxidants present in *P. multiflorum* roots are mostly hydrophilic compounds such as 2,3,5,4'-tetrahydroxystilbene 2-O-β-D-glucopyranoside (Wang, et al., 2013). They are easily extracted using water and can be released more efficiently from the root using hot water treatment like decoction. In addition, the anthraquinone content in *P. multiflorum* root may be converted into free anthraquinones form such as emodin during boiling in decoction method. This emodin, is a well-known antioxidant compound and it is also responsible for the antioxidant activity of *P. multiflorum* (Lin, et al. 2010).

5.5 Correlation

In this study, all extracts were exhibited different TPC, TFC, and EC_{50} values due to different phytochemicals composition present in the extracts respectively. As mentioned earlier, the TPC and TFC determined were contributed to the antioxidant activity of all herb extracts. This can be further explained by determining the correlation between TPC, TFC, and EC_{50}. As shown in Table 4.5, a strong positive correlation was determined between TPC and TFC ($r = 0.884$), whereas EC_{50} showed a strong negative correlation with TPC ($r = -0.896$) and TFC ($r = -0.904$), respectively. The strong positive correlation between TPC and TFC indicates that they were correlated with each other in which that is an increase in TFC when TPC was increased. This is because flavonoid is a subgroup of phenolic compounds (Iqbal, Salim and Lim, 2015). Furthermore, a strong negative correlation was observed for the EC_{50} with TPC and TFC. This suggests that the present of phenolic compounds and flavonoids are contributed to the antioxidant activity or free radicals scavenging activity of the herb extracts

with lower EC_{50} values. This was in agreement with Chai and Wong, (2012), the authors found that the EC_{50} were negatively correlated with phenolic compounds and flavonoids. Nevertheless, the presence of other phytochemicals such as polysaccharides and other organic compounds may also be contributed to the antioxidant properties of the herbs (Yi, Liu and Dong, 2013). Hence, phenolic compounds and flavonoids play a key role in antioxidant properties of the herb extracts but they are not the only metabolites that influence the antioxidant activity.

5.6 Limitations of Study

There were few unavoidable limitations found in this study. Firstly, some of the data were obtained in high standard deviations due to insufficient time to repeat those slightly imperfect data. In addition, the cost of tested herb samples was relatively high, and some of the antioxidant tests cannot be carried out due to limited budgets available to purchase chemical reagents.

5.7 Further Studies and Suggestions

In future study, numerous assays such as oxygen radical absorbance capacity (ORAC), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) cation decolorization assay, hydroxyl radical scavenging activity, ferric-reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), and metal chelating activity can be used to further analyze the antioxidant properties and antioxidant activities of the herb extracts prepared by different methods (Sowndhararajan and Kang, 2013). Moreover, more herbs

should be tested in the same manner to evaluate the effects of different preparation methods on their antioxidant properties.

Besides, a study can be conducted in order to identify the types of phytochemicals present in all herb extracts. Techniques like high performance liquid chromatography (HPLC) can be employed to isolate, purify and identify individual components in different herb extracts which have contributed to the antioxidant activity. Furthermore, an *in vivo* study may be conducted using animal models to further evaluate the effects of antioxidant properties and activities of traditional Chinese medicinal herbs prepared by different methods.

CHAPTER 6

CONCLUSION

In this study, phytochemicals of two traditional Chinese medicinal herbs (*L. barbarum* and *P. multiflorum*) were extracted using decoction and grounded methods. According to the results obtained, crude extract of *L. barbarum* prepared by grounded method showed the highest TPC and TFC values as well as the lowest EC_{50} value among the extracts of *L. barbarum*. On the other hands, crude extract of *P. multiflorum* prepared by decoction method showed the highest TPC and TFC values not only among the extracts of *P. multiflorum* but also included the extracts of *L. barbarum*. It also showed the lowest EC_{50} value, which exhibited the highest DPPH free radical scavenging activity among all of the extracts. Strong negative correlations were determined by EC_{50} with TPC and TFC. As a conclusion, both *L. barbarum* and *P. multiflorum* were considered as good sources of antioxidants since all extracts showed high amount of TPC and TFC, with low EC_{50} determined in DPPH assay. It is recommended to prepare the *L. barbarum* using grounded method and prepare the *P. multiflorum* using decoction method to obtain the greatest antioxidant properties of the herbs.

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Appendix A

SPSS Output A: Statistical analysis of extraction yield percentage (%) of crude extracts obtained

ANOVA

Extraction yield percentage (%) compared between crude extracts of *L. barbarum* and crude extracts of *P.multiflorum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.195	1	6.195	13.324	.011
Within Groups	2.790	6	.465		
Total	8.985	7			

ANOVA

Extraction yield percentage (%) compared between crude extracts prepared by decoction method and crude extracts prepared by grounded method

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.420	1	2.420	2.212	.188
Within Groups	6.565	6	1.094		
Total	8.985	7			

Appendix B

SPSS Output B: ANOVA and Tukey test of TPC results of *L. barbarum* extracts

ANOVA

TPC of *Lycium barbarum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3818.654	2	1909.327	632.607	.000
Within Groups	45.273	15	3.018		
Total	3863.926	17			

TPC of *Lycium barbarum*

Tukey HSD

Preparation methods	N	Subset for alpha = 0.05		
		1	2	3
Decoction	6	24.2315		
Extract pulveres	6		28.6347	
Grounded	6			57.0945
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix C

SPSS Output C: ANOVA and Tukey test of TPC results of *P.multiflorum* extracts

ANOVA

TPC of *Polygonum multiflorum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1849.994	2	924.997	49.728	.000
Within Groups	279.018	15	18.601		
Total	2129.012	17			

TPC of *Polygonum multiflorum*

Tukey HSD

Preparation methods	N	Subset for alpha = 0.05		
		1	2	3
Grounded	6	45.5883		
Extract pulveres	6		55.0858	
Decoction	6			70.2078
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix D

SPSS Output D: ANOVA and Tukey test of TFC results of *L. barbarum* extracts

ANOVA

TFC of *Lycium barbarum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	58844.048	2	29422.024	980.410	.000
Within Groups	450.149	15	30.010		
Total	59294.196	17			

TFC of *Lycium barbarum*

Tukey HSD

Preparation methods	N	Subset for alpha = 0.05		
		1	2	3
Decoction	6	26.2500		
Extract pulveres	6		37.5595	
Grounded	6			152.7976
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix E

SPSS Output E: ANOVA and Tukey test of TFC results of *P.multiflorum* extracts

ANOVA

TFC of *Polygonum multiflorum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	51133.252	2	25566.626	902.837	.000
Within Groups	424.771	15	28.318		
Total	51558.023	17			

TFC of *Polygonum multiflorum*

Tukey HSD

Preparation methods	N	Subset for alpha = 0.05		
		1	2	3
Grounded	6	38.3036		
Extract pulveres	6		95.9524	
Decoction	6			168.5714
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix F

SPSS Output F: ANOVA and Tukey test of EC_{50} of *L. barbarum* extracts

ANOVA

EC_{50} of *Lycium barbarum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	218436.187	2	109218.093	15713.857	.000
Within Groups	104.256	15	6.950		
Total	218540.443	17			

EC_{50} of *Lycium barbarum*

Tukey HSD

Preparation methods	N	Subset for alpha = 0.05		
		1	2	3
Grounded	6	34.1891		
Decoction	6		215.4682	
Extract pulveres	6			297.9253
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix G

SPSS Output G: ANOVA and Tukey test of EC_{50} of *P.multiflorum* extracts

ANOVA

EC_{50} of *Polygonum multiflorum*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	85775.017	2	42887.509	15400.104	.000
Within Groups	41.773	15	2.785		
Total	85816.790	17			

EC_{50} of *Polygonum multiflorum*

Tukey HSD

Preparation methods	N	Subset for alpha = 0.05		
		1	2	3
Decoction	6	24.2215		
Extract pulveres	6		68.3370	
Grounded	6			187.6445
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix H

SPSS Output H: Independent samples T-test for TPC assay

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Decoction method	Equal variances assumed	4.014	.073	-19.395	10	.000	-45.97633	2.37050	-51.25814	-40.69453
	Equal variances not assumed			-19.395	5.479	.000	-45.97633	2.37050	-51.91310	-40.03957
Grounded method	Equal variances assumed	.395	.544	7.477	10	.000	11.50617	1.53878	8.07755	14.93478
	Equal variances not assumed			7.477	7.992	.000	11.50617	1.53878	7.95710	15.05523
Extract pulveres	Equal variances assumed	3.533	.090	-15.744	10	.000	-26.45117	1.68005	-30.19456	-22.70778
	Equal variances not assumed			-15.744	7.797	.000	-26.45117	1.68005	-30.34296	-22.55938

Appendix I

SPSS Output I: Independent samples T-test for TFC assay

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Decoction method	Equal variances assumed	6.368	.030	-44.554	10	.000	-142.32143	3.19438	-149.43896	-135.20390
	Equal variances not assumed			-44.554	7.461	.000	-142.32143	3.19438	-149.78133	-134.86153
Grounded method	Equal variances assumed	1.913	.197	33.996	10	.000	114.49405	3.36783	106.99005	121.99805
	Equal variances not assumed			33.996	8.143	.000	114.49405	3.36783	106.75148	122.23661
Extract pulveres	Equal variances assumed	.140	.716	-21.157	10	.000	-58.39286	2.76001	-64.54253	-52.24318
	Equal variances not assumed			-21.157	9.681	.000	-58.39286	2.76001	-64.57007	-52.21564

Appendix J

SPSS Output J: Independent samples T-test for DPPH assay (EC_{50} compared between two herb extracts with the same preparation method)

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Decoction method	Equal variances assumed	143.303	.000	186.624	10	.000	191.24671	1.02477	188.96337	193.53004
	Equal variances not assumed			186.624	5.033	.000	191.24671	1.02477	188.61766	193.87575
Grounded method	Equal variances assumed	7.136	.023	-130.364	10	.000	-153.45539	1.17713	-156.07820	-150.83259
	Equal variances not assumed			-130.364	5.152	.000	-153.45539	1.17713	-156.45471	-150.45608
Extract pulveres	Equal variances assumed	4.040	.072	147.224	10	.000	229.58832	1.55945	226.11365	233.06298
	Equal variances not assumed			147.224	5.100	.000	229.58832	1.55945	225.60312	233.57352

Appendix K

SPSS Output K: Independent samples T-test for DPPH assay (EC_{50} of extracts compared with EC_{50} of ascorbic acid)

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
EC_{50} of *Lycium barbarum* (decoction)	Equal variances assumed	159.708	.000	-201.503	10	.000	-206.18151	1.02322	-208.46138	-203.90164
	Equal variances not assumed			-201.503	5.003	.000	-206.18151	1.02322	-208.81134	-203.55168
EC_{50} of *Lycium barbarum* (grounded)	Equal variances assumed	81.766	.000	-171.923	10	.000	-24.90235	.14485	-25.22509	-24.57962
	Equal variances not assumed			-171.923	5.138	.000	-24.90235	.14485	-25.27170	-24.53300
EC_{50} of *Lycium barbarum* (extract pulveres)	Equal variances assumed	5.125	.047	-186.001	10	.000	-288.63857	1.55181	-292.09622	-285.18092
	Equal variances not assumed			-186.001	5.001	.000	-288.63857	1.55181	-292.62735	-284.64980

EC_{50} of *Polygonum multiflorum* (decoction)	Equal variances assumed	2.597	.138	-243.737	10	.000	-14.93480	.06127	-15.07133	-14.79828
	Equal variances not assumed			-243.737	5.818	.000	-14.93480	.06127	-15.08588	-14.78373
EC_{50} of *Polygonum multiflorum* (grounded)	Equal variances assumed	9.575	.011	-152.648	10	.000	-178.35775	1.16843	-180.96116	-175.75433
	Equal variances not assumed			-152.648	5.002	.000	-178.35775	1.16843	-181.36090	-175.35459
EC_{50} of *Polygonum multiflorum* (extract pulveres)	Equal variances assumed	11.608	.007	-378.607	10	.000	-59.05025	.15597	-59.39777	-58.70274
	Equal variances not assumed			-378.607	5.119	.000	-59.05025	.15597	-59.44840	-58.65211