PARAMETERS THAT EFFECT THE ANTIOXIDANT PROFILE AND PHENOLIC COMPOUND FROM *CENTELLA ASIATICA* EXTRACT

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by

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LIST OF SYMBOL

Symbol		Unit
%1	Percentage of inhibition of DPPH activity	%
A_o	Absorbance of control	-
A_1	Absorbance of sample	-
IC_{50}	Concentration of inhibitor (reduced by half)	μg/mL
A	Total phenolic contents	mgGAE/g
C	Concentration of gallic acid	mg/mL
V	Volume of solvent	mL
m	Mass of extract	mg

LIST OF ABBREVIATION

C. asiatica	*Centella Asiatica* (Pucuk Pegaga)
FCR	Folin-Ciocalteu Reagent
Rotavap	Rotary Evaporator
Na_2CO_3	Sodium Carbonate
$C_{30}H_{48}O_7S$	Triterpenoids
$C_{48}H_{78}O_{19}$	Asiaticosides
UV-Vis	Ultraviolet-visible spectrophotometer
DPPH	2,2-diphenyl-1-picrylhydrazyl
OD	Optical Density
HPLC	High-Performance Liquid Chromatography

FAKTOR-FAKTOR YANG MEMBERI KESAN KEPADA PROFIL ANTIOKSIDAN DAN SEBATIAN FENOLIK DARIPADA EKSTRAK *CENTELLA ASIATICA*

ABSTRAK

Centella asiatica selalunya digunakan sebagai rempah dan herba dalam perubatan kerana mempunyai kandungan antioksidan dan jumlah kandungan fenolik yang sangat tinggi. Air, metanol dan etanol telah digunakan bagi menganalisis data mengenai aktiviti antioksida dan kandungan fenolik. Etanol mempunyai jumlah aktiviti DPPH yang paling tinggi pada jam yang ke 7 iaitu 65.06 % (IC_{50}=291.00 mg/ml) diikuti oleh air yang berjumlah 63.48 % (IC_{50}=408.26 mg/ml) dan metanol dengan jumlah 62.39 % (IC_{50}=515.86 mg/ml). Data yang diperolehi telah dibandingkan dengan lengkungan piawai BHT bagi mengenalpasti pelarut yang terbaik berdasarkan aktiviti DPPH. Pertambahan aktiviti DPPH boleh menyebabkan pertambahan dari segi nilai TPC. Etanol telah menunjukkan hasil TPC yang paling tinggi diikuti dengan air dan metanol dengan nilai masing-masing berjumlah 228.57 mgGAE/g, 115.63 mgGAE/g dan 64.41 mgGAE/g. Ini kerana etanol mempunyai kumpulan hidrosi yang kuat berbanding metanol dan air. Data tersebut telah dibandingkan dengan jumlah lengkungan piawai asid galik. Sekiranya masa bertambah, jumlah fenolik dalam *C. asiatica* juga bertambah, kerana kapasiti ekstrak bergantung pada masa pelarut. Komponen aktif di dalam *C. asiatica* ialah *asiaticoside*. Masa penahanan sebanyak 2.60 min dikesan menggunakan HPLC manakala ukuran piawai sebanyak 2.75 min. Hasil yang diperolehi menunjukkan etanol adalah pelarut terbaik untuk pengekstrakan komponen aktif mengandungi aktiviti antioksida dan kandungan fenolik bagi *C. asiatica* berbanding metanol dan air.

PARAMETERS THAT EFFECT THE ANTIOXIDANT PROFILE AND PHENOLIC COMPOUND FROM *CENTELLA ASIATICA* EXTRACT

ABSTRACT

Centella asiatica is commonly used as a spice and medicinal herb due to its antioxidant activity and total phenolic contents. Water, methanol and ethanol were used to analyze the amount of antioxidant activity and total phenolic contents. Ethanol extraction at 7 hr showed the highest value of DPPH scavenging activity with 65.06 % (IC_{50}=291.00 mg/ml) followed by water with 63.48 % (IC_{50}=408.26 mg/ml) and methanol with 62.39 % (IC_{50}=515.86 mg/ml). The data from the experiment had been compared with the BHT standard curve in order to finalised the best solvent based on DPPH scavenging activity. The increase in DPPH scavenging activity contributes by high amount of TPC. Ethanol gave the highest TPC followed by water and methanol at 228.57 mgGAE/g, 115.63 mgGAE/g and 64.41 mgGAE/g, respectively. This is because ethanol has stronger hydroxyl group compared to methanol and water. The results were compared with the standard curve of gallic acid. As the time increase the phenolic compound from *C.asiatica* also increase, this is because the phenolic content contributed by capacity of extract due to the time of extraction. The active component that contributed to the phenolic compound is asiaticoside. As The retention time using HPLC was 2.60 min while the standard time was 2.75 min. The result showed that ethanol is the best solvent for extraction of active component with antioxidant activity and phenolic contents of *C. asiatica* compared to methanol and water.

CHAPTER ONE

INTRODUCTION

1.1 Background of *Centella Asiatica*

Centella can be made up of almost 50 species and one of them is *Centella asiatica (C. asiatica). Centella asiatica* is a tropical medicinal plant from *Apiaceae* (*Umbelliferae*) family that commonly used in medical field (James and Dubery, 2009). This plant also have another names such as bua-bog, tiger herbal and gotu kola. All of their parts are useful for medical field. It is also being used as traditional drug in order to cure wound, decrease blood pressure, heal bruises and diuretic (Ullah, 2009). In Malaysia, *Centella asiatica* known as "pegaga" commonly used for wound healing. It is said to have antioxidant properties that very important to cure diseases. Antibacterial and antifungal properties and also anticancer and antioxidant properties can be found from the extracts of this plant (James and Dubery, 2009; Orhan et al., 2013; Hashim et al., 2011). The main compound in the *C. asiatica* is triterpenoid.Triterpenoid contains asiatica acid, madecassic acid, madasiatic acid, asiaticoside and madecassoside can be extracted using soxhlet extraction with different solvents. Water, methanol and ethanol are used as the solvent for this experiment. As bioactive phenolic compounds responsible for the antioxidant activity, several procedures was conducted to determine and evaluated the profiles of antioxidant and phenolic compound from *C. asiatica* extract.

1.2 Problem Statement

C. asiatica is found to be extremely safe and shown great potential to be used in biomedical applications. Asiaticoside is one of the major bioactive constituents in *C. asiatica* which contributes to the antioxidant and anti-inflammatory activities. The quality of active constituents in *C. asiatica* is dependent on the extraction processes. There are several conventional processes such as microwave assisted extraction, ultrasonic assisted extraction and soxhlet extraction that could be used for extraction that could be used for extraction of the active compounds from *C. asiatica*. Comparing to the other conventional extraction processes, soxhlet extraction cost considerably less, easier to setup and can give high efficiency of extraction. Hence, in this study, soxhlet extraction was used for extraction of active compound from *C. asiatica*.

Type of solvents used need to be considered as there are many solvents can be used for this experiment. Green solvents such as water, methanol and ethanol were used but they can contributed the different amount of antioxidant activity and also phenolic contents.

It is known that some active compounds could degrade when exposed to heat at long duration of time. Futhermore, the extraction of these active compounds are dependent on the type of solvent. These inherently alter the quality of extracts. With regard to soxhlet extraction of *C. asiatica*, not much has been reported on the effect of extraction time and solvent type on the quality of the extracts.

1.3 Research Objectives

The aim of this research is to understand the effect of extraction time and solvent type on the quality of the extract. Here, the quality of extract was determined from the antioxidant activity and total phenolic contents. In short, the specific objectives of the current study are:

i) To understand the effects of the solvent on *C. asiatica* extract obtained using soxhlet extraction.

ii) To determine and evaluate the antioxidant activity of *C. asiatica* extracts.

iii) To investigate and evaluate the total phenolic content of *C. asiatica* extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Centella Asiatica*

Long ago plants have been used as foods or medicine in almost every parts of the world. Plants have primary and also secondary metabolites that very importants for them. Secondary metabolites in plants serve as plant defence mechanisms attacks by microorganisms, insects and herbivores. Plant also contains phenolic compounds that can served as natural antioxidant. Nowadays, focus on plant research has increased all over the world (Rattanakom and Yasurin, 2014).

Centella asiatica is a small, slender, tender and faintly aromatic herb (Saxena and Pal, 2016). It is a tropical medicinal plant from *Apiaceae* (*Umbelliferae*) family native to Southeast Asian countries such as India, Sri Lanka, China, Indonesia, and Malaysia as well as South Africa and Madagascar (Jamil et al., 2007). *C. asiatica*, commonly known as Gotu kola, Mandukparni, Asiatica pennywort, Indian pennywort, Indian water navelwort, wild violet, and tiger herb, is one of the tropical plants that have fan-shaped, green leaves that are harvested and used for medicinal purposes and known as "*pegaga*" in Malaysia (Orhan, 2012). This herb can achieved an height up to 15 cm and the leaves which is orbicular-renniform shape can have 1.5-5 cm and 2-6 cm long (Zahara et al., 2014).

Each parts of *C. asiatica* have their own specialities such as for the roots. This is because this part rich in amino acids which are serine, alanine, glutamic, threonine, aspartic, histidine and lysine (Dora and Jyoti, 2011; Kulsoom Zahara et al., 2014). This parts also rich in retinol, riboflavin, ascorbic acid, thiamine, niacin and carotene as well as they are very benefit to human (Dora and Jyoti, 2011). Countries such as Turkey and

China since centuries had being using *C. asiatica* due to the medical benefits. This is because it can help in increasing the skin strength which has been weaken by polluted environment. Figure 2.1 shows, the typical *C. asiatica*.

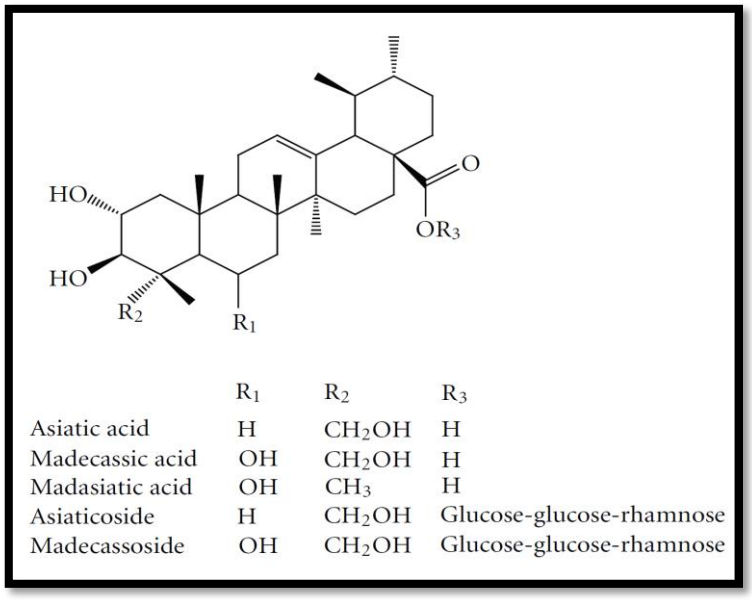
Figure 2.1 The picture of *Centella asiatica*

C. asiatica is used as anti-bacterial, anti-inflammatory, anti-diabetic, anti-oxidant, antifungal, skin soothing effect, skin pigmentation relief and promoting skin elasticity. *C. asiatica* is very famous herb that very valuable in Ayurvedic medicine. Ayurveda knowledge which is known as Medhya Rasayana useful for brain tonic. It can revitalize nerve and brain cells, increases memory and concentration because it has an overall rejuvenating effects on our body. As for the Chinese traditional medicinal knowledge, it has anti-aging properties for skin and tightens older skin.

This plant is used for prevention of wrinkles and for rejuvenation of tissues (Agme-Ghodke et al., 2016). It can be used in cream to get rid of cane blemishes as it can helps the skin maintain collagen in the dermal layer of our tissues for a long time . Ayurvedic medicine usually used for treatment of leprosy, psoriasis, fevers, insanity, fatigue, asthma, respiratory infection, ulcers, eczema, skin tuberculosis, wounds, stomach aches, arthritis, hepatitis, colds, varicose veins and high blood pressure that had been reported by Rattanakom and Yasurin (2014) because healers have used it as to

treat a variety of ailments. It also can helps in get rid of excessive sebum by gives a balanced amount of water to our body.

Centella asiatica contain a lots of number of compounds which is belonging to different classes. The chemical constituents of centella asiatica are triterpenoids, asiaticosides and polyacetylenes. Triterpenoid also known as saponin is the major chemical class that can be found in this plant that act as primary constituents that mainly believed to be responsible for its wide therapeutic actions and the major triterpene saponoside derivatives found in *C. asiatica* as shown in Figure 2.2.



	R_1	R_2	R_3
Asiatic acid	H	CH_2OH	H
Madecassic acid	OH	CH_2OH	H
Madasiatic acid	OH	CH_3	H
Asiaticoside	H	CH_2OH	Glucose-glucose-rhamnose
Madecassoside	OH	CH_2OH	Glucose-glucose-rhamnose

Figure 2.2 The major triterpene saponoside derivatives found in *Centella asiatica* (Orhan, 2012)

This chemical compound can cause alteration in gene expression in human fibroblast (Coldren et al., 2003; Lu et al., 2004). Asiaticoside is useful in human dermal fibroblast as it can induce the type I callogen synthesis of human (Lee et al., 2006). The main active components of *C. asiatica* are two glycosides which are asiaticoside and madecassoside and their respective genins are asiatic acid and medecassic acid shown in Figure 2.3.

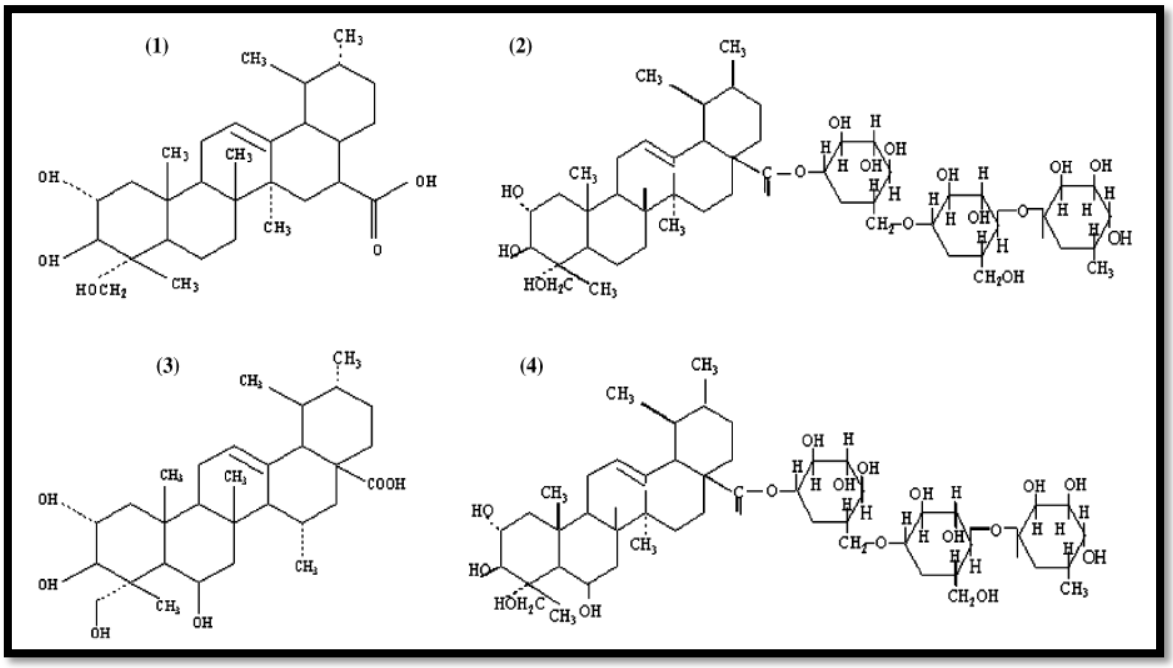


Figure 2.3 The chemical structure of Asiatic acid (1), Asiaticoside (2), Madecassic acid (3), and Madecassoside (4) (Kim et al., 2009)

The significant differences in active constituent contents had being used for the standardization of this species as described in European Pharmacopoeia which is be observed that the sample of *C. asiatica* from different countries such as India and Madagascar shown the different contents in active components (Das and Mallick, 1991). Madagascar region shows the highest level of asiaticoside compared to other regions (Randriamampionona et al., 2007).

Brahmic acid, centellin, centellicin, asiaticin, bayogenin, terminolic acid, 3*β*,6*β*,23- trihydroxyolean-12-en-28-oic acid, 3*β*,6*β*,23-trihydroxyurs- 12-en-28-oic acid, 3-O-[*α*-L-arabinofuranosyl] 2*α*,3*β*,6*β*,23-*α* tetrahydroxyurs-12-en-28-oic acid, centellasapogenol A, centellasaponins A-D, ursolic acid, pomolic acid, 3-epimaslinic acid, 23-O-acetylmadecassoside, and 23-O-acetylasiaticoside B are some other of active compounds that exists in *C. asiatica* as triterpene (Orhan, 2012).

2.1.1 Active Components of *Centella Asiatica*

Centella asiatica is one of the important herbs that very useful in medical field as it has a lots of bioactive components. Active compounds in *C. asiatica* are very unique as each of the components have their own benefits. The main of the chemical constituents exist in *C. asiatica* are asiaticosides, triterpenoids and polyacetylenes. Figure 2.4 below shows the diagram of chemical constituents in *C. asiatica*.

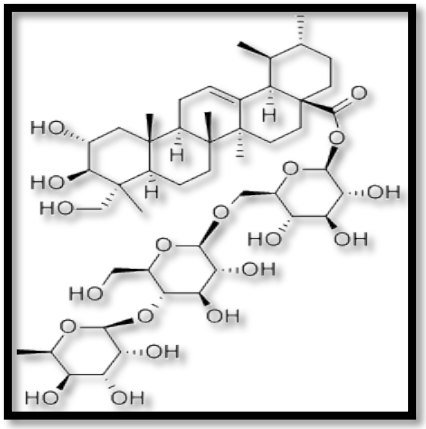


Figure 2.4 Chemical structure of asiaticoside of *Centella asiatica*

Asiaticosides has the molecular formula which is $C_{48}H_{78}O_{19}$ and 959.12 g/mol of molecular weight have a lots of benefits. It is the terpenoid saponin component that isolated from *C. asiatica* that useful in antioxidant and anti-inflammatory activities because it have very strong wound-healing properties and it is also reported can reduces scar formation. Asiaticoside reported can reduced the allergic inflammation by decreased the intracellular calcium (Jiang et al., 2017). The roots part of *C. asiatica* reported to have the highest amount of asiaticoside genin which is asiatic acid (Zainol et al., 2008).

The other study, shows that the leaves of the *C. asiatica* contains high in both asiaticoside and madecassoside components between 2.6% and 6.42% dry weight (Randriamampionona et al., 2007; James and Dubery, 2009).

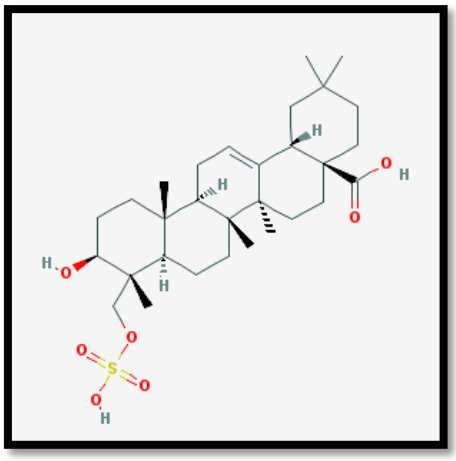


Figure 2.5 Chemical structure of triterpenoid of *Centella asiatica*

Figure 2.5 shows, the chemical structure of triterpenoid of *C. asiatica* that having molecular formula and molecular weight which are $C_{30}H_{48}O_7S$ and 552.767 g/mol respectively. The most common name is triterpenoid saponins which is a glycoside with triterpenoid aglycone structures and widely found in nature. It is the largest group of natural compounds that synthesized from two five-carbon building blocks that have a lots of classes and almost 40,000 different of terpenoids found in plants species (Rabi and Bishayee, 2009; Rohdich et al., 2005; Withers and Keasling, 2007).

This active component is useful to treat various types of cancer. As one of the major causes of death for female species which is breast cancer becomes hot topic for nowadays. Many experiments had already done in order to cure this disease but not all of the experiments found that breast cancer can been treated. Asian countries posses various of the pharmacological properties that very useful to treat this cancer by having the herbs as the cure. The active component in *C. asiatica* which is triterpenoids can give the high potential agents for chemoprevention and therapy of breast cancer if human take this herb as their daily diets (Bishayee et al., 2011; Dennis et al., 2009). The triterpenoids content in centella asiatica are known to exhibit cytotoxicity against a

variety of tumor cells as well as anticancer efficacy and also reduce the risk of infections by stimulating the immune system (Bishayee et al., 2011).

Polyacetylenes can be found in *Umbelliferae, Araliaceae* and *Asteraceae* families and reported more than 1400 different of polyacetylenes and their related bioactive components have been isolated from plants (Christensen and Brandt, 2006). More than 50 classes of polyacetylenes group that can be found in the plants species but there are only 14 types of polyacetylenes classes that can be found in *Umbelliaferae* (*Apiaceae*) family such as *C. asiatica*. One of them is liphatic C_{17}-polyacetylenes such as falcarinol and falcarindiol and they are widely found in *Umbelliferae* and *Araliaceae* family and this bioactive components have received a lots of attention due to their boundless physiological impact, including anticancer, antifungal and so on (Christensen and Brandt, 2006; Lin et al., 2016).

Figure 2.6 shows, the group of aliphatic acetylenes isolated from utilized parts of Umbelliaferae food plants.

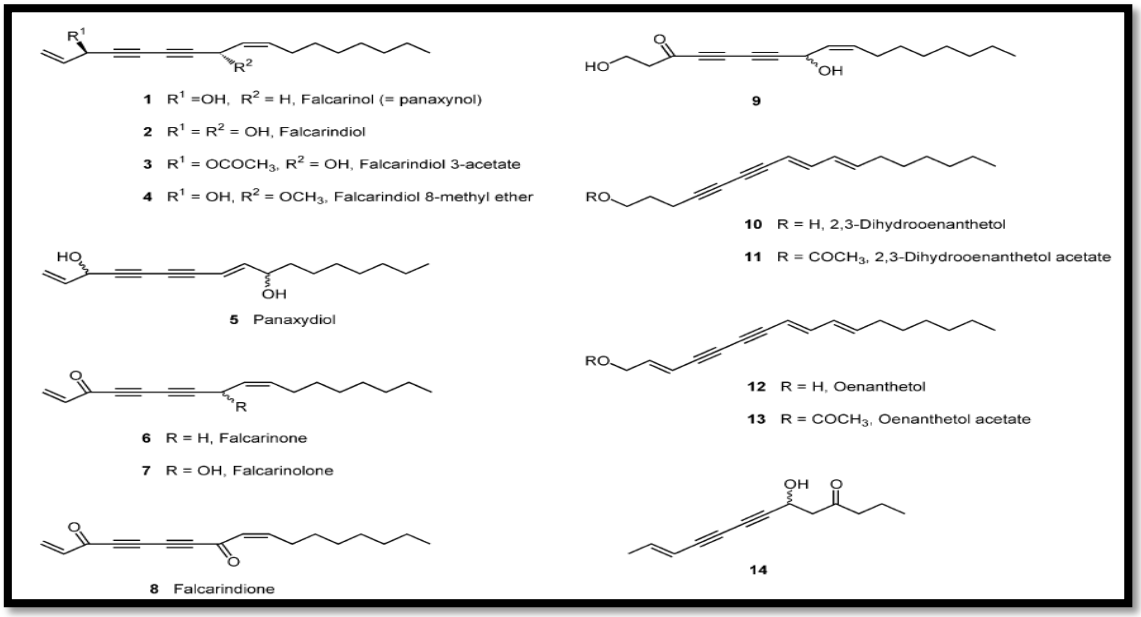


Figure 2.6 The group of Aliphatic acetylenes isolated from utilized parts of *Umbelliaferae* food plants (Christensen and Brandt, 2006)

Usually, polyacetylic compunds can be found from the underground parts of *C. asiatica* which is their roots (Zahara et al., 2014). Although it have a vast of benefits, but still need to take precaution steps in using it as a medicine. Some of polyacetylenes have been reported undesirable due to their toxic properties that can causes allergic reaction in normal tissue after exposure (Lin et al., 2016; Huang et al., 2011). *Apiaceae* family have been used as traditional medicine in Asia countries such as China, Malaysia and Indonesia in order to treat influenza, fever, malaria and menstrual disorders.

2.1.2 Antioxidant Properties of *Centella Asiatica*

The antioxidants is the substance that is able to neutralize reactive molecules and reduce oxidative damages. Results of metabolic processes and environmental sources. The antioxidative activity of plant parts is mainly contributed by the active compounds present in them. Since the growing trend of replacing the synthetic antioxidants by natural ones become great interest in both academics and the food industry many studies and research on antioxidative properties of essential oils and various extracts from many plants were conducted (Pittella et al., 2009). *Centella asiatica* is a herb that has a high antioxidant activity. There are many methods in order to increase the shelf life of foods and the addition of antioxidant is one of the methods. The antioxidative activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. Many phenolic compounds, particularly flavonoids, exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic and vasdilatory actions (Cook and Samman, 1996).

The content of antioxidant in *C. asiatica* is very high and has very good potential to be explored as source of natural antioxidants compared to the antioxidant activity of other herbs such as rosemary and sage (Jaswir et al., 2004). Scavenging DPPH free radical is one of the methods that can identify the antioxidant activity of *C. asiatica*.

The DPPH is a very stable radical and is widely used in order to assess the radical scaveging activity of antioxidant compounds. DPPH will be reduced in the solution of methanol with the presence of a hydrogen. Formation of non-radical in DPPH-H4 will cause the hydrogen to be a donating agent. The result of this transformation is the color change from purple to yellow, which is measured spectrophotometrically (Dewi and Maryani, 2015). Figure 2.7 shows, the chemical reaction mechanism for DPPH.

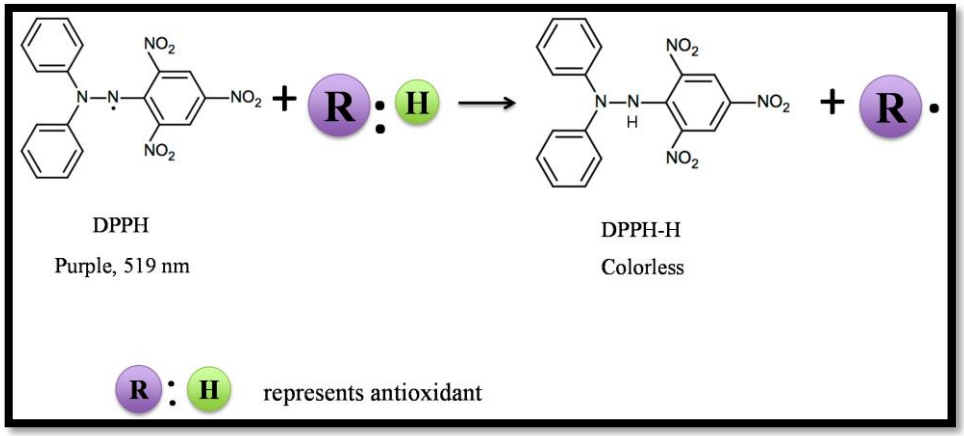


Figure 2.7 Reaction mechanism of DPPH with antioxidant. R:H = Antioxidant Radical Scavengers; R = Antioxidant Radical (Liang and Kitts, 2014)

Another study stated that antioxidant content in *C. asiatica* (84%) is comparable to vitamin C (88%) and grape seed extract (83%) (Hashim et al., 2011). In other study, it is also reported that they find out the ascorbic acid, total carotene, beta carotene and total phenolics are major source that we can found in *C. asiatica*. Their regression analysis showed that the relationship between antioxidant activity and antioxidant contents was highly significant (Gupta and Prakash, 2009). *C. asiatica* originated from India shows they have high antioxidant contents and activities (Subhasree et al., 2009). Among three solvents which are water, ethanol and light petroleum used to extract *C. asiatica* at 25ºC for 24 hours, the highest antioxidant can be found in ethanol extract followed by water. As for light petroleum, they observed that it yielded negative antioxidant activities. Ethanolic extract shows the highest antioxidant activity compared

to metholic extract and water extraction. There was no significant different in total phenolic content between ethanolic and metholic extract (Rattanakom and Yasurin, 2014).

Generally antioxidant are used to stops the chain reaction by changing the nature of free radical. The oxidative diseases can cause :

i) A chemically reactive oxygen free radical attacks fatty acids, DNA, proteins or chlolesterols moleculs forming other free radicals.

ii) Destruction chain reaction.

iii) The injury to tissues and the formation of more free radicals.

iv) Damage to the cell membrane lipids and proteins which is disabling them.

v) Precancerous change the DNA.

vi) Oxidation of blood chlolesterol initiating steps leading to heart disease.

vii) An untimately diseases and tissues aging such as cancer, heart diasease, aging and macular degeneration can occur.

2.1.3 Phenolic Compound of *Centella Asiatica*

The three major compounds of *C. asiatica* are triterpenoids, asiaticosides and polyacetylenes. These components of *C. asiatica* are useful such as for asiaticosides are very useful as antileproic agents. In 2005, different of solvents had been used in order to find out the chemical profile of plant material by extracting the polyphenols (Pinelo et al., 2005). Triterpenoid acids, volatile and fatty (Subban et al., 2008), alkaloids, glycosides (Jamil et al., 2007), flavonoids and steroids (Subban et al., 2008) have been isolated from the different parts of the *C. asiatica* (Harwoko et al., 2014). Other than that, *C. asiatica* also contain a lots of derivatives of triterpene and it were identified as asiatic acid and madecassic acid. Both of them have their own heterosides named which are asiaticoside and madecassoside and reported these two triterpene derivatives constituting almost 10% of the plants (Orhan, 2012).

Flavonoids one of the major compounds that can be found in the plants and it can be classes into many categories (Bhandari et al., 2007), and other than flavonoids are polyacetylenes and phenolic acids (Subban et al., 2008). Different solvents and methods can cause the different amount of extraction yields (Esmaeilzadeh et al., 2014), thus antioxidant activity and chemical profile also different. By using different of solvent the extraction of essential oil had been studied and UV-Vis and HPLC were used as the equipment to find out the chemical constituents inside of *C. asiatica* (Rattanakom and Yasurin, 2014).

From research study, ethanol extraction give greater yield of extract (7.3 %) (w/w) followed by methanol (5.0 %) and water (3.3 %). It shows that, the high concentration of ethanol can give high value of diameter zone of inhibition (Idris and Nadzir., 2017) . This is because, ethanol is more polar compared to water and methanol. Ethanol got a hydroxyl head group so it is stronger meaning it can extract neutral lipids and polar lipids. Hydroxyl group which is it can permeate the cell wall. The organic solvent will diffuse into the solid material and later on it will stabilized the compound as they solubilize the compounds with the similar polarity.

2.2 Extraction Method of *Centella Asiatica*

Extraction is the initial and most important step in the recovery and purification of bioactive compounds from plant materials. Several types of extraction process can be done in order to extract bioactive components content in centella asiatica such as Soxhlet Extraction, Microwave Assisted Extraction (MAE), Ultrasonic Assisted Extraction (UAE) and Maceration Extract. Soxhlet and maceration process known as traditional methods of extraction as well as MAE and UAE are modern and latest methods for extraction of bioactive components. MAE and UEA required an expensive equipments while soxhlet, maceration and fractionation does not require any expensive equipments (Halfadji et al., 2013).

Different types of extraction could give different value of products yield but timing and costing are important although it can give the results instantly. Soxhlet extraction will gave high yield compared to maceration and fractionation process but MAE and UAE can gave high yield of products and save time when conducting the experiment (Halfadji et al., 2013; Murugan and Parimelazhagan, 2014). Although they can yield more products, soxhlet extractor lowest in capital cost.

2.2.1 Extraction Methods

Extraction methods can be catogorized into traditional and modern methods. Traditional method which are soxhlet and maceration extract (ME) while microwave assisted extraction (MAE) and ultrasonic assisted extraction (UAE) are modern methods.

MAE utilizes microwave energy to facilitate partition of analytes from the sample matrix into the solvent (Trusheva et al., 2007). The microwave radiation interacts with dipoles of polar and polarizable materials (Azwanida, 2015). They also mentioned that MAE can be considered as selective methods that favour polar molecules and solvents with dielectric constant as ethanol has the lowest dielectric constant compared to methanol and water (Azwanida, 2015).

Solvent	Dielectric constant (20°C)
Hexane	1.89
Toluene	2.4
Dichloromethane	8.9
Acetone	20.7
Ethanol	24.3
Methanol	32.6
Water	78.5

Figure 2.8 Dielectric constant of some commonly used solvents (Kaufmann and Christen, 2002)

Gallo et al. (2010) mentioned that the efficiency of extraction of bioactive compounds obtained with the microwave extraction process was in general about four times higher than that resulting from ultrasonication process. They also stated that MAE provide significant advantages in terms of extraction efficiency and time savings. MAE could be used as an effective method to extract antioxidant components considering factors such as the extraction time and the solvent wastage (Gallo et al., 2010). The

results showed, that the antioxidant activity measured using MAE was higher compared to UAE. Azwanida (2015) stated that this method is limited to small-molecule phenolic compounds such as gallic acid, isoflavin and quacertin.

UAE also known as sonication extraction as it is use ultrasound ranging from 20 kHZ to 2000 kHZ (Handa et al., (2008). Ultrasound can increased the surface contact between solvents and samples and permeability of cell walls and enhancing mass transport of the solvents into the plant cells (Azwanida, 2015; Dhanani et al, 2017). The reduction in extraction time and solvent consumption can be achieved by using UAE but ultrasound more than 20 kHZ can effect on the active components through the formation of free radicals (Azwanida, 2015; Kaufmann and Christen, 2002; Handa et al, 2008). Trusheva et al. (2007) mentioned that UAE most effective methods as it gave high yield, reduce extraction time between 10 min to 30 min and high selectivity.

ME is a method use in wine making but widely used in medicinal plants research. This method involed soaking plant samples in a stoppered container with a solvent and allowed to stand at room temperature for certain time with frequent agitation (Handa et al., 2008). It will break the plant's cell wall to release the soluble phytochemicals (Azwanida, 2015). It is the easiest and simple method but the large volume of solvents is used and need proper management.

2.2.2 Soxhlet Extraction

A Soxhlet extractor is a piece of laboratory apparatus that made from Pyrex glass and it is well known apparatus used to extract organic compounds from a solid matrix (Jensen, 2007; Behring, 2018). It is invented by a German chemist, Franz Ritter von Soxhlet (1848-1926) in 1878 who is tried to separate the fats from milk solids (Jensen, 2007; Behring, 2018).

This extraction process only can be used when the desired compound had limited solubility in solvent and not all of the organic extraction can be used for the soxhlet extraction. Soxhlet extractor can be designate into several parts as shown in Figure 2.9 below.

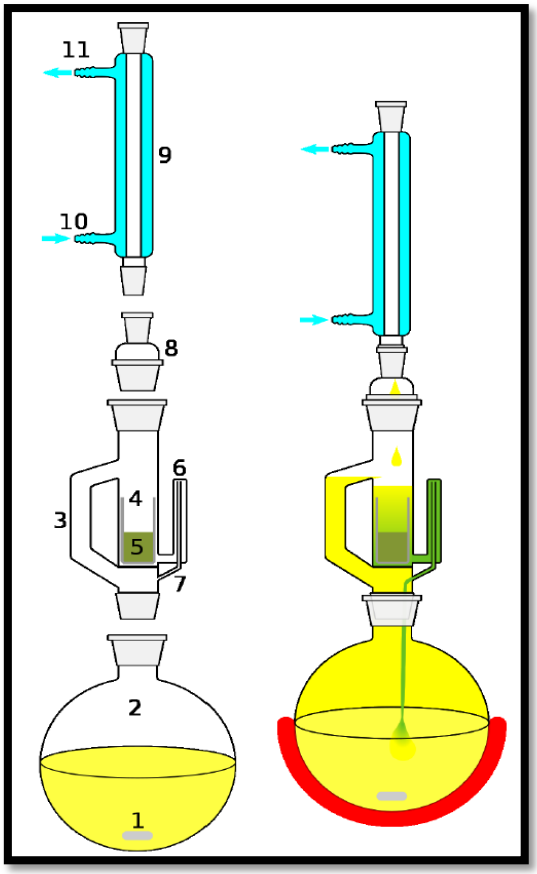


Figure 2.9 A schematic representation of a Soxhlet extractor (Jensen, 2007)

Stirrer bar(1), still pot(2), distillation path(3), thimble(4), solid(5), siphon top(6), siphon exit(7), expansion adapter(8), condenser(9), cooling water in(10) and cooling water out(11) are the important parts for soxhlet extractor. Soxhlet's extraction tube is a open at both top and bottom and consists two side arms. Three important parts of the soxhlet extractor are condenser(top), extractor or thimble(middle) and boiling flask(bottom). A condenser, thimble and boiling flask are another three parts that will attached with soxhlet extraction tube for extraction process to occured. A condenser will be filled with flowing colling medium such as water in order to condense the vapor into liquid (Behring, 2018). A porous thimble is a place where we insert our desire solid for extract their organic compounds and the boiling flask is placed beneath the extractor will filled with the solvent. The solid should be grinded first before insert it into the thimble and boiling flask should not be overfilled and the volume of the solvent should be 3 to 4 times volume of the soxhlet chamber (Jensen, 2007).

The solvent filled in the boiling flask is boils at certain temperature which is the boiling for solvent used in this extraction process. As it reach it boiling point, it will vaporized through one side arm of the extractor later on, the cooling medium will cooled down the temperature so that it will condesed back to the liquid state. The liquid will drips down through the middle part which is extractor part that contained the solid matrix. Solid-liquid contact will occured as the liquid passing through the solid and extracting organic compounds. Once the extract solution reaches another side of the arm then, it will drained back into the boiling flask and the process continues until it reach the desired time for the extraction (Behring, 2018).

Some limitations of soxhlet method such as exposure to hazardous of the solvent used during extraction process and it is considered not environmental friendly. This is because, it can contribute to the pollution problem and it is also limited to a dry and

finely solid matrix. Another disadvantages are solvent-sample ratio, temperature and agitation speed need to be considered for this method (Azwanida, 2015; Amid et al., 2010).

2.2.3 Parameters that Effect the Extraction Process

Extraction is the initial and the most important step in the recovery and purification of bioactive compounds from the plant materials. The extraction method can be affected by various conditions of parameter that effected the total antioxidant content and total phenolic content such as type of solvent used, extraction time, temperature and sample to solvent ratio. Many researchers have been studied this operating condition for various plant, fruit and food.

One of the effects that influenced the extraction of antioxidant activity is the solid to solvent ratio. The effect of solid to solvent ratio on total antioxidant content and total phenolic content had been studies on plant extract (Wong et al., 2013). The results from their studies shown that the best parameter and condition that were needed to be used for the plant or herbs extract so it can give the highest yield of antioxidant activity and phenolic content was solid to solvent ratio of 1:20 (w/v) (Wong et al., 2013). While study by Tan et al., (2011) mentioned that high solid to solvent ratio could promote an increasing the concentration gradient which will increase diffusion rate that allow greater extraction of solid by solvent.

According to Frank et al., (1999) a solid solubility is affected by change in the activity coefficient which varies with the temperature and composition of the solution. In conclusion, the extraction solid to sample ratio, the antioxidant content can be affected by solid to solvent ratio.

As the antioxidant activity is affected by the extraction of solvent used, Al-Farsi and Lee (2008) stated that the diffusion rate was increased and allowed greater extraction of the solid by the solvent when the high solid to solvent ratio was used that resulting the increasing of concentration gradient. The yield of the antioxidant activity and DPPH radical scavenging on *C. asiatica* based on the study by (Wong et al., 2013) will increased by increasing the solid to solvent ratio.

The time of extraction and temperature can affected the extraction of antioxidant from plant. Analysis of some studies shows that extraction temperature between 60°C to 80°C were most efficient for extraction of dry herbs by giving the highest total antioxidant content and result obtained from DPPH assay also gave the highest value on that operating temperature (Reihani et al., 2016; Maja et al., 2012).

Azwanida (2015) stated that boiling C. asiatica at 90°C will increased phenolic contents and antioxidant activity. This is because heat can enhance the recovery of antioxidant capacity and phenolic compound in the extraction (Durling et al., 2007). The optimum extraction time and temperature for antioxidant compounds varies with antioxidant capacity. The rate of extraction of thermally stable the antioxidant at elevated temperature is higher than the rate of decomposition of less soluble antioxidant (Chandrika and Fereidoon, 2005).

Durling et al. (2007) mentioned that the increasing in extraction temperature could degrade phenolic compounds. This is because chemical and enzymatic degradation can cause the interference compound stability and reduced the extraction efficiency. Finally, Cacace and Mazza (2003) stated that increasing in extraction temperature will caused the increasing the extraction rate and reduced the extraction time.

The most suitable solvent systems for the extraction is water/ethanol mixtures due to the different polarities of bioactive components (Maja et al., 2012). Based on the studies of Cacace and Mazza (2006) and Kumoro et al., (2009) the increasing in temperature could accelerated rate of reaction and been reported that increased in temperature up to 74°C also could increased the extracted phenolics quantity but it also induces compound degradation process. Increasing in boiling temperature and time resulting that the increased significantly of total antioxidant activity and total phenolic content (Al-Farsi and Lee, 2008; Ong et al., 2010).

2.3 Analytical Techniques / Analysis

Methods of data collection and methods of analysis are linked together. To do this effectively requires the use of certain types of data collection methods which involved special data analysis techniques.

The qualitative analysis and quantitative analysis were importants because with qualitative analysis we can determined what chemical compounds exists in the sample and quantitative analysis we can determined what amount of the compounds are included in the sample. These sample may develop impurities at various stages of their development and can cause disturbance or problem during the experiment.

Analytical techniques can be classify into three type of categories which are chromatography, spectroscopy and electrophorosis. Different types of analytical analysis can give different value of product yield although the result we can get instantly. Chromatography is an analytical technique based on the separation of molecules due to the differences in structures or composition.

High-Performance Liquid Chromatography (HPLC) had been used in this experiment in order to detect the active compounds in *C. asiatica*. This is because HPLC can give a powerful separation method that be able to resolve the components in a mixture with a large number similar analytes (Imran et al., 2015).

CHAPTER THREE

METHODOLOGY

3.1 Materials and Equipments Used

The experiment were conducted using several types of materials and equipments such as *C. asiatica* (dried), ascorbic acid, beakers, DPPH (solid), absolute ethanol, absolute methanol, ethanol (HPLC grade) were purchased from Merck Chemicals Ltd. (United Kingdom), asiaticoside was purchased from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile (HPLC grade), tap water, fume board, measuring cylinder, fridge, spatula, FCR, gallic acid, laboratory weighing scale, Soxhlet extractor equipment, lab oven, micropipette, cuvettes, Buchi rotary evaporator, sodium carbonate, test tubes, UV-Vis spectrophotometer, conical flasks and volumetric flasks.

3.2 Methods

3.2.1 Preparation of the Sample

Centella asiatica was purchased from area of Bagan Serai, Perak. The whole parts of *C. asiatica* such as stems, roots and leaves were used as the samples. The samples were washed with excess water to remove dirt and soil that might be attached to it. The initial weight of the samples was recorded. Drying process with air-oven as it can give higher phenolic composition as well as antioxidant composition after extraction (Zainol et al., 2008).

Then, the sample was separated on the tray and placed into the oven under sufficient operating condition which is temperatures between 50^{o}C to 60^{o}C within several hours until it reached constant weight at 5% moisture content (Darfour et al., 2014; Idris and Nadzir., 2017). In order to find out the moisture content, the mass of the samples was weighted every two hours. If the weight of the samples remain contant,