ANTIOXIDANT ACTIVITY OF *Cosmos caudatus* EXTRACTS BY USING DIFFERENT TYPES OF EXTRACTION METHODS

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SUPERVISOR'S DECLARATION

"I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)."

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STUDENT'S DECLARATION

I declare that this thesis entitled "Antioxidant Activity of *Cosmos caudatus* Extracts by Using Different Types of Extraction Methods" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : …….....

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Date : …….....

In the name of ALLAH, Most Gracious, Most Merciful

Dedicated to my beloved parents

Mohd Khairi Bin Ayob & Nooraishah Binti Ariffin

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ANTIOXIDANT ACTIVITY OF *Cosmos caudatus* EXTRACTS BY USING DIFFERENT TYPES OF EXTRACTION METHODS

ABSTRACT

The discoveries on new resources of natural antioxidant from plants have attracted increasing interest due to high potential of antioxidant capacity. In this paper, antioxidant activity of *Cosmos caudatus* extracts was investigated by using different types of extraction methods which were maceration extraction, Soxhlet extraction and ultrasonic-assisted extraction using 70% acetone (v/v) as solvent. The antioxidant activity and ascorbic acid content from each method was determined by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay and High Performance Liquid Chromatography (HPLC) respectively. The results show that the three extraction methods exhibit significant value of ascorbic acid content. Ultrasonic-assisted extraction was the efficient method as it exhibits the highest amount of ascorbic acid content with 26.590 mg ascorbic acid equivalent per 1 g sample (mg AAE/g) with the corresponding value of 42.208% of its antioxidant activity after 60 minutes extraction. This is followed by Soxhlet extraction (13.527 mg AAE/g) and maceration extraction (5.190 mg AAE/g) within 8 hour and 24 hour extraction time respectively. Analysis by HPLC also shows that *C. caudatus* extract using ultrasonic-assisted extraction has the highest vitamin C content (2.440 mg AAE/g) followed by Soxhlet extraction (0.968 mg AAE/g) and maceration extraction (0.739 mg AAE/g). For further study, optimization of the ultrasonic extraction method is proposed to achieve the optimum condition in extracting bioactive compounds from *C. caudatus*.

ANTIOKSIDAN AKTIVITI *Cosmos caudatus* EKSTRAK DENGAN MENGGUNAKAN PELBAGAI TEKNIK PENGEKSTRAKAN

ABSTRAK

Penemuan sumber baru antioksidan semulajadi daripada tumbuh-tumbuhan semakin menarik minat disebabkan potensi kapasiti antioksidan yang tinggi. Di dalam kajian ini, aktiviti antioksidan daripada ekstrak *C. caudatus* telah dikaji dengan menggunakan pelbagai jenis kaedah pengekstrakan iaitu pengekstrakan pengerapan, pengekstrakan Soxhlet dan pengekstrakan ultrasonik dengan menggunakan 70% aseton (v/v) sebagai pelarut. Aktiviti antioksidan dan kandungan asid askorbik dari setiap kaedah ditentukan oleh 2.2-Diphenyl-1-Picrylhydrazyl (DPPH) dan Kromatografi Cecair Prestasi Tinggi (HPLC). Keputusan menunjukkan bahawa tiga kaedah pengekstrakan mempamerkan nilai signifikan di dalam kandungan asid askorbik. Pengekstrakan menggunakan ultrasonik adalah kaedah yang cekap kerana ia mempamerkan kandungan asid askorbik tertinggi dengan jumlah 26.590 mg asid askorbik per 1 g sampel (mg AAE/g) hanya dengan 42.208% aktioksidan aktiviti dalam 60 minit pengekstrakan. Ini diikuti oleh pengekstrakan Soxhlet (13.527 mg AAE/g) dan pengekstrakan pengerapan (5.190 mg AAE/g) dalam jam 8 dan 24 jam pengekstrakan. Analisis oleh HPLC juga menunjukkan bahawa *C. caudatus* diekstrak oleh pengekstrakan ultrasonik mempunyai kandungan vitamin C tertinggi (2.440 mg AAE/g) diikuti oleh pengekstrakan Soxhlet (0.968 mg AAE/g) dan pengekstrakan pengerapan (0.739 mg AAE/g). Kajian selanjutnya mencadangkan menggunakan kaedah pengekstrakan yang berpotensi seperti pengekstrakan mikrogelombang dan pengoptimuman parameter kaedah ini dalam menemui kaedah pengekstrakan yang cekap dalam mengekstrak sebatian bioaktif daripada *C. caudatus.*

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LIST OF ABBREVIATIONS

A	- Absorbance
AAC	- Ascorbic acid content
AAE	- Ascorbic acid equivalent
AEAC	- Ascorbic acid equivalent antioxidant capacity
C. caudatus	- *Cosmos caudatus*
CSE	- Conventional solvent extraction
DPPH	- 2,2-Diphenyl-1-Picrylhydrazyl
DW	- Dry weight
FRAP	- Ferric reducing antioxidant potential
HPLC	- High Performance Liquid Chromatography
KH_2PO_4	- Potassium dihydrogen phosphate
LLE	- Liquid-liquid extraction
ME	- Maceration extraction
SE	- Soxhlet extraction
SOD	- Superoxide Dismutase
UAE	- Ultrasonic-assisted extraction

LIST OF SYMBOLS

°C	- Degree Celcius
%	- Percentage
(v/v)	- volume (of solute) per volume (of solvent)
(w/v)	- weight (of solute) per volume (of solvent)
µg	- microgram
µl	- microlitre
mg	- miligram
g	- Gram
kg	- Kilogram
kHz	- kilo Hertz
M	- Molarity
mAU.s	- milliabsorbance units
min	- minute
ml	- mili Litre
L	- Litre
mm	- millimeter
nm	- nanometer
rpm	- Revolution per minute
W	- watt

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Various techniques of extraction have been developed over past few decades for extraction process. The development is done in order to shorten the extraction time, decrease the solvent consumption, increase the extraction yield, and enhance the quality of extracts (Wang and Weller, 2006). According to Gao and Liu (2005), the most frequently used extraction techniques employ conventional solvent extraction, Soxhlet extraction, and ultrasound-assisted extraction. For instance, employing of these different extraction techniques can be seen through the numerous studies on antioxidant activities of plants (Kalia *et al.*, 2008; Wang *et al.*, 2010; Jun *et al.*, 2011).

Antioxidant can be defined as compound that is capable of slowing or preventing the oxidation process of the molecules by inhabiting the initiation of oxidizing chain reactions. Generation of free radicals during metabolism and other activities exceed the antioxidant capacity of a biological system gives rise to

oxidative stress. Oxidative stress plays a role in the aging process. This concept is supported by increasing evidence that oxidative damage plays a role in age-related degenerative diseases, and that the dietary antioxidants oppose this and lower risk of disease (Krishnaiah *et al.*, 2007).

Most of the antioxidants in the market nowadays are manufactured synthetically and knows as synthetic antioxidant. This synthetic antioxidant is widely used in edible vegetable oil and cosmetics (Qin et *al.*, 2009). However, according to toxicologists and nutritionists, the side effects of these synthetic antioxidants have already been documented. For example, these substances had shown carcinogenic effects in living organisms (Ghomi et *al.*, 2010).

Increasing awareness of community on effects of this synthetic antioxidant made considerable attention has been paid to natural antioxidant from plants. The consumption of herbs containing antioxidants has been reported to provide protection against a wide range of degenerative disease such as cancer and ageing (Duyn and Pivonka, 2000). *Cosmos caudatus* or its local name “ulam raja” is one of the herbs that can be found in most tropical regions. Generally, *C. caudatus* was used in traditional medicine to improving blood circulation and as anti-aging due to its pharmacological activity. The pharmacological activity is attributed to its phytochemical property such as antioxidant yet has a potential as a natural antioxidant source.

1.2 Problem Statement

Demands of antioxidant nowadays are in great demand due to community awareness towards free radicals or called “oxidative cells” that can cause human cancer and other diseases. However, most of the antioxidant in market is being produced synthetically and side effects of these synthetic antioxidants have already been documented. Therefore, new alternative of findings natural antioxidant is crucial to overcome this problem such as *C. caudatus* that can be considered as a highly nutritious plant.

Common extraction methods like conventional solvent extraction and steam distillation has disadvantage such as low yields, formation of by-products, and the presence of toxic organic residues in the extracts. The drawbacks linked to these techniques have led to the searching for new alternative extraction processes. This study was conducted in order to introduce and compare various extraction techniques such as maceration extraction, Soxhlet extraction, and ultrasonic-assisted extraction as a new alternative applicable method to extract antioxidant from *C. caudatus.*

1.3 Research Objective

The main objective of this research is to investigate the effect of different types of extraction methods on the recovery of antioxidant compounds from *C. caudatus* extracts.

1.4 Scope of the Study

The scopes have been drawn in order to achieve the research objective:

1.4.1. Studying the effect of extraction methods on extracting antioxidant compounds from *C. caudatus*. The extraction methods used are maceration extraction, Soxhlet extraction, and ultrasound-assisted extraction.

1.4.2. Determining the antioxidant activity from *C. caudatus* extracts by using DPPH assay.

1.4.3 Identifying the ascorbic acid content from *C. caudatus* extracts by using HPLC.

1.5 Significance of the Study

Extraction of *C. caudatus* has highly beneficial effects as antioxidant and can give big impact in nutraceuticals and food chemistry. By demonstrating antioxidant activity, it has potential as a new source of natural antioxidant. In addition, it will also benefit future studies in determining other herbs that has same potential as a new alternative of natural antioxidant for human benefits.

Due to high demand for antioxidant in the pharmaceutical and food processing, it is important for the industries to have simple, rapid and efficient extraction. These studies focus on the best methods in producing high antioxidant content capacity instead of conventional method to fulfill increment demand for antioxidant in the future.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, topics that related to this study will be explained in detail based on previous literature. Generally, the subchapter that covered in this chapter are *Cosmos caudatus* (ulam Raja), antioxidants and extraction methods. The others parts that related in this study also briefly explained.

2.2 *Cosmos caudatus*

Cosmos caudatus or its local name “ulam raja” is an annual, short-lived, perennial, aromatic herb originated from tropical Central America and belongs to the family *asteraceae*. It is an edible plant having about 20-26 species worldwide. According to study by Shui *et al.,* (2005), *C. caudatus* was first been introduced to Philippines via the Spaniards. It grows wild and widespread in almost tropical regions such as in Mexico, United States, South America, Thailand and Malaysia. They can be recognized by its physical properties which is bearing purple, pink, white, yellow florets that can grows up about 1-8 ft. tall, sparsely hairy and leaves that finely dissected, 10 - 20 cm long (Rasdi *et al.,* 2010). While in terms of its chemical contents, *C.caudatus* contains 0.3% of proteins, 0.4% of fats and carbohydrates.

For Malaysian, young leaves of this herb was widely popular as herbal salad which often eaten raw with rice and chili, shrimp paste or “budu” (fermented anchovies) to enhance flavours. Meanwhile, in a traditional medicine, it was used to strengthen the bones which can act as prevention to osteoporosis, improving blood circulation, anti-aging agent, reducing body heat, promote fresh breath and to treat infections associated with pathogenic. In other hand, in scientifically several biological activities in *C. caudatus* have been reported. For instance, *C.caudatus* contains 101.20 ± 5.62 μg/g DW of total carotenoid, 37.44 ± 0.50 μg/g DW of lutenin and 63.76 ± 1.01 μg/g DW of β-carotene (Fatimah *et al.,* 2012). Besides that, previous study by Ragasa *et al.,* (1999) reported that several antimutagen and antifungal compounds which are cotunolide, stigmasterol, lutein and 4,4′-bipyridine was found in *C. caudatus*. In addition, compounds isolated from

C.caudatus also has been claims as a good sources of antioxidant compounds and were found had extremely high antioxidant capacity of about 2400 mg/L ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh sample (Abas *et al.*, 2006; Shui *et al.*, 2005).

Figure 2.1 *Cosmos caudatus*

2.3 Free Radicals

Oxidation metabolism occur continuously in the human body and food system as part of normal cellular function and cause formation of free radicals. Free radicals can be defined as high energy unstable atoms with unpaired electron and very reactive (Fang *et al.,* 2002). This undesirable metabolic by product are normally generated by internal and external factors. Internal factors are through normal part of metabolism within the mitochondria, inflammation processes, physical exercises, and stress hormones. Meanwhile the external factors is by smoking, environmental pollutants, drugs, and ozone. The unstable free radicals have tendency in reducing their energy level by transferring excess electron to any adjacent substances. According to Wong *et al.,* (2006), excess free radicals production attack nearby tissue by oxidizing membrane lipids, cellular proteins and DNA that can cause cellular damage which lead to the development of chronic diseases. Likewise, the interaction of oxygen free radicals with of lipid portion of body leads to formation of new radicals such as hydroperoxides, superoxide, lipoid oxides and hydroxyl radical whose type may interact with biological systems in a cytotoxic ways (Aytul, 2010).

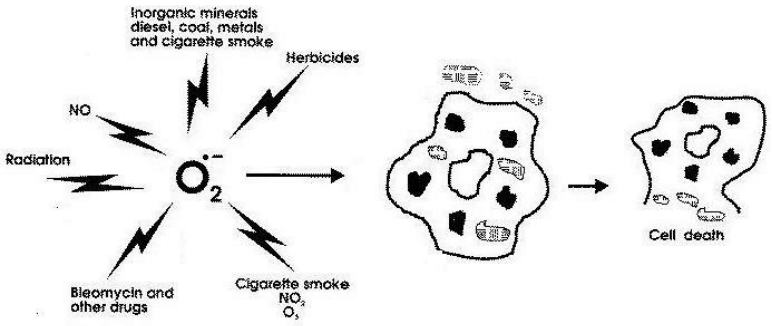

Figure 2.2 Effect of free radicals (Aytul, 2010)

2.4 Antioxidant

Antioxidant can boost cellular defences and help to prevent oxidative damage to cellular components. Antioxidant can be defined as compound that is capable of slowing or preventing the oxidation process of the molecules by inhabiting the initiation of oxidizing chain reactions. Another definition is given by Halliwell and Gutteridge (1995), which is "any substance that when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate". Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent where free radicals is produced which initiate the chain reactions. Presence of antioxidants terminated these chain reactions and inhibits other oxidation reactions by removing free radical intermediate and being oxidized themselves respectively.

The antioxidant system can be divided into two major groups, enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants or endogenous antioxidants is a type of antioxidants that been produced within human system body which consist of primary and secondary enzymatic defences. The primary enzymatic defences consists of three enzymes which is glutathione peroxidase, catalase and superoxide dismutase which responsible in neutralizing the free radicals. Meanwhile secondary enzymatic defences consist of glutathione reductase and glucose-6-phosphate dehydrogenase that functions to reduces oxidized substances to its reduced form creating a reducing environment (Ratnam *et al.,* 2006). However, endogenous antioxidants system does not suffice to maintain free radical at low concentration.

According to Pietta (2000), human body system need to depend on various types of antioxidants that present through diet to maintain free radical concentrations at low levels. This is because endogenous antioxidants system itself is incomplete without exogenous antioxidants. Therefore, non-enzymatic oxidants or called exogenous antioxidants are needed in order to disequilibrium redox state in favor of oxidation. Exogenous antioxidants can be obtained from dietary allowance either natural or synthetic drugs such as selenium, enzyme cofactors, nitrogen compounds, polyphenols and vitamins.

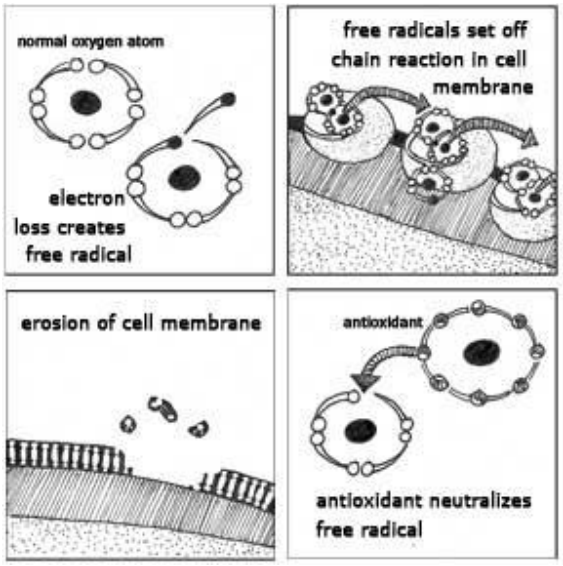

Figure 2.3 Antioxidants mechanism in combating free radicals (Aytul, 2010)

2.4.1 Natural and Synthetic Antioxidants

Generally, there are two types of antioxidant which is synthetic and natural antioxidants. Synthetic antioxidant is a compound of antioxidant that being synthesized by chemicals reaction. Employing of synthetic antioxidants such as nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ), 2,4,5-trihydroxybutyropenone (THBP), propyl gallate (PG), hydroxymethylphenol (IONOX-100) and butylated hydroxyl toluene (BHT) is widely used in industrial processing food, dietary supplements, and cosmetics to prolong the storage stability, prevent rancidification and high performance (Guan *et al.,* 2005; Guo *et al.,* 2006). Even though they are high performance in protecting product quality, excess antioxidant might produce toxicities or mutagenicity and thus endanger for humans. Pursuant to Mukhopadhyay (2006), BHT is made from paracresol and isobutylene by chemical reactions which have unnatural molecular structures that may turn out to be hazardous. BHT use in rat feed caused them to develop fatal haemorrhages in the plural and peritoneal cavities in organs such as pancreas (Fki *et al.,* 2005). On the other hand, BHT also being reported to encourage the development of tumors from previously initiated cells (Malkinson, 1999).

Nevertheless synthetic antioxidants have been widely used in food industry as described previously, the trend search for antioxidant from natural sources has attracted much attention as an alternative of synthetic antioxidants. This is supported by past studies that shown many herbs and plants have potential as new natural source of antioxidant and exhibited stronger antioxidant activity (Cai *et al.,* 2003; Shui *et al.,* 2005; Siddhuraju and Becker, 2003; Kruawan and Kangsadalampai, 2006; Kalia *et al.,* 2008; Gan *et al.,* 2010). Natural antioxidant is a compound that

can be naturally found in vegetables, fruits and plants which are safe to consume. The compound of this natural antioxidant can be essential oils, phenolic compounds (tocopherol, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyl derivatives, amino acids, and amines), carotenoids as well as ascorbic acid (Shaiban *et al.*, 2006). They have capability of preventing diseases without gives a long term side effects. For instance, Tseng *et al.,* (1998) reported that Thai herbs can inhibit the mutagenesis induced by various chemical in animal experiment.

2.4.2 Ascorbic Acid

Flavonoids, melatonin, retinoids (Vitamin A), tocopherols (Vitamin E) and ascorbic acid (Vitamin C) are examples of antioxidants that derived naturally and found in most herbs and plants. Ascorbic acid or vitamin C is a water soluble organic compound that involved in many biological processes. They play an important role in electron transport, hydroxylation reactions and oxidative catabolism in animal metabolism (Velisek and Cejpek, 2007). Ascorbic acid compound is a monosaccharide oxidation-reduction catalyst which can reduce, hence neutralize the reactive oxygen species such as hydrogen peroxide (H_2O_2). Finding by Fang *et al.,* (2002) states that ascorbic acid exhibits protective effect against free radical–induced oxidative damage. The ascorbic acid neutralizes free radicals nearby body cells, thus preventing cellular damage and slows down the aging process.

The main sources of natural ascorbic acid mostly found in fruits and vegetables such as citrus fruits, strawberries, peppers, tomatoes, cabbage, spinach (Davey *et al.,* 2000). Presence of ascorbic acid is crucial in collagen formation, a protein that gives structure to bones, cartilage, muscle, and blood vessels, aids in the absorption of iron, and helps maintain capillaries, bones, and teeth. It is reported that ascorbic acid may prevent the formation of *N*-nitroso compounds, which are potentially mutagenic and cause gastric cancer risk (Correa, 1992; Byers and Guerrero, 1995). Figure 2.4 shows chemical structure of ascorbic acid.

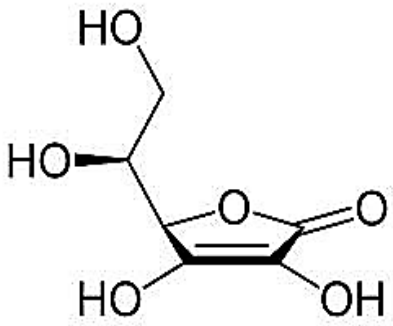

Figure 2.4 Chemical structure of ascorbic acid

2.5 Extraction Principles

Extraction is the process of separation of active substances of plant or animal from a mixture by a mechanical or chemical action. An extraction procedure is one that separate one constituent phase from another (Geankoplis, 2003, p. 776). During an extraction process one or more compounds or solutes are transferred from one liquid phase to another immiscible liquid phase through mixing. Generally, there are two forms of extraction process; liquid-liquid extraction and solid-liquid extraction.

Liquid-liquid extraction was generally performed where both of the compounds is in liquid phase. Liquid–liquid extraction (LLE) also known as solvent extraction and partitioning, where compounds was being separated based on their relative solubility in two different immiscible liquids; commonly water and organic solvent (Weinstein *et al.,* 1998). LLE also referred as the separation process involving mixture substance by using suitable solvent. In laboratory scale, LLE ordinarily performed using separatory funnel. An example of LLE is such as production of organic compounds, perfume processing and biodiesel.

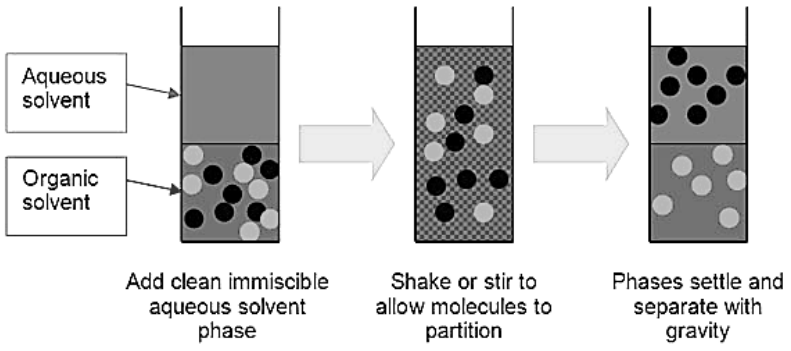

Figure 2.5 Liquid-liquid extraction (Liquid-liquid extraction, 2012)

Meanwhile, solid-liquid extraction is a process that separates the desired solute constituent or to remove an undesirable solute component from the solid phase by contacting the solid with the liquid phase or solvent. The solvent may be in single component liquid or a mixture. The contacting results in diffusing of solutes from the solid phase to the liquid phase hence separate the components that are originally in the solid. In extraction, there are two main steps. The first step is contacting of the solvent with the solid to transfer the soluble constituent or solute to the solvent and the second step is separation of the residual solid from solution. The complete process includes the separate recovery of the solutes and solvent to obtain pure product which is done by another unit operation such as evaporator or distillation. Therefore, in this study solid liquid extraction was employed since the plant material used is in solid form.

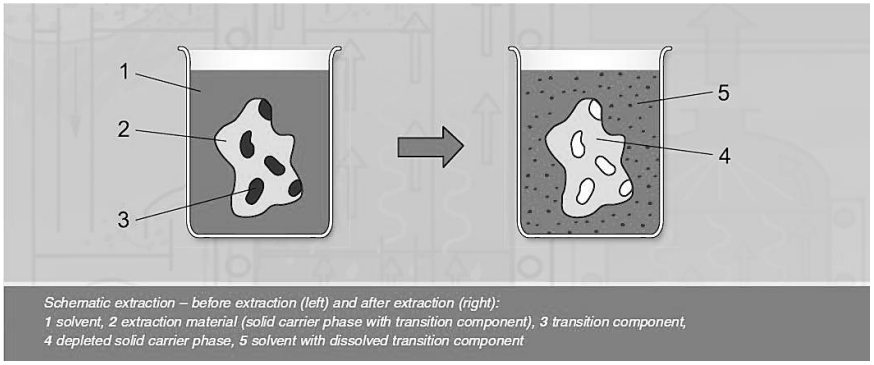

Figure 2.6 Solid-liquid extraction

2.6 Extraction Methods

Various extraction techniques have been developed and employed for extraction of active ingredients from plant materials such as conventional solvent extraction, Soxhlet extraction, ultrasonic-assisted extraction, microwave-assisted extraction and supercritical fluid extraction (Wang and Weller, 2006; Xi *et al.*, 2010). These novel techniques are developed to fulfil the increment demand by industry in order to shorten extraction time, decrease the solvent consumption, increase pollution prevention, increase the extraction yield and quality of products. In this study, method use is only limited up to three extraction methods which are maceration extraction, Soxhlet extraction, and ultrasonic-assisted extraction. Table 2.1 below shows different extraction method by different studies.

Table 2.1 Comparison of yield of different extraction methods for selected plants

Plants	Extraction methods	Parameter	Yield	References
Citrus paradisi	ME	Solid to sample ratio: 1:5 Temperature: 25°C Time: 180 min Solvent: 70% ethanol	13.5 (g/kg)	Giannuzzo *et al.*, (2003)
	SE	Solid to sample ratio: 1:10 Temperature: 80°C Time: 480 min Solvent: 70% ethanol	15.2 (g/kg)	

****** ME, maceration extraction; SE, Soxhlet extraction; UAE, ultrasonic-assisted extraction

Table 2.1 Comparison of yield of different extraction methods for selected plants (continued)

Plants	Extraction methods	Parameter	Yield	References
Pinus radiate Bark	ME	Solid to sample ratio: 1:10 Temperature: 40°C Time: 360 min Solvent: 70% acetone	49.5 (mg/g)	Aspe and Fernandez (2011)
	SE	Solid to sample ratio: 1:10 Time: 360 min Solvent: 70% acetone Solvent: *n*-hexane	47.2 (mg/g)	
	UAE	Solid to sample ratio: 1:10 Temperature: 25°C Time: 12 min Solvent: 70% acetone	62.1 (mg/g)	
Caraway seeds	ME	Solid to sample ratio: 1:20 Temperature: 69°C Time: 60 min Solvent: *n*-hexane	13.38 (mg/g)	Chemal *et al.,* (2004)
	SE	Solid to sample ratio: 1:20 Temperature: 69°C Time: 300 min Solvent: *n*-hexane	16.28 (mg/g)	
	UAE	Solid to sample ratio: 1:20 Temperature: 69°C Time: 60 min Solvent: *n*-hexane	14.45 (mg/g)	

****** ME, maceration extraction; SE, Soxhlet extraction; UAE, ultrasonic-assisted extraction

Table 2.1 Comparison of yield of different extraction methods for selected plants (continued)

Plants	Extraction methods	Parameter	Yield	References
Potentilla atrosanguinea Lodd.	ME	Solid to sample ratio: 1:10 Temperature: 27°C Time: 1440 min Solvent: 50% ethanol	17.2 (mg/g)	Kalia *et al.*, (2008)
	SE	Solid to sample ratio: 1:30 Temperature: 80°C Time: 480 min Solvent: 50% ethanol	24.2 (mg/g)	
	UAE	Solid to sample ratio: 1:10 Temperature: 35°C Time: 60 min Solvent: 50% ethanol	18.8 (mg/g)	
Amaranthus caudatus	ME	Solid to sample ratio: 1:20 Temperature: 25°C Time: 1440 min Solvent: methanol	76.32(mg/g)	Bruni *et al.*, (2002)
	UAE	Solid to sample ratio: 1:20 Temperature: 25°C Time: 60 min Solvent: methanol	63.7 (mg/g)	
Rose hip seeds	UAE	Solid to sample ratio: 1:25 Temperature: 69°C Time: 60 min Solvent: *n*-hexane	32.5 (mg/g)	Szenimihalyi *et al.*, (2002)

****** ME, maceration extraction; SE, Soxhlet extraction; UAE, ultrasonic-assisted extraction

2.6.1 Maceration Extraction

2.6.1.1 Principles and Mechanisms

Maceration extraction is one of the oldest techniques employing in solid-liquid extraction over century. According to Thovogi (2009), the maceration mechanism involves leaving the pulverized plant to soak in a suitable solvent in a closed container. Simple maceration usually was performed at room temperature by mixing the raw material with the solvent with certain solid to solvent ratio. The mixture is leaving for several days with occasional shaking or stirring to enhance extraction process. Closed container is necessary in maceration extraction due to longer period extraction time that may causes loss of solvent volume (Bhavani, 2010). Maceration technique usually related to winemaking process where the phenolic materials of the grape; tannins, anthocyanin and flavour compounds are leaches from the grape skin, seeds and stems into the must. Employing of maceration extraction can be seen through previous study in extracting sea buckthorn (Michel *et al.,* 2011); extraction from *Pinus radiate* Bark (Aspe and Fernandez, 2011); and naringin from *Citrus paradisi* (Giannuzzo *et al.,* 2003).

2.6.1.2 Advantages and Disadvantages of Maceration Extraction

The advantages behind this conventional methods is simple operation and easy to carry out (Gao and Liu, 2005). Besides that, this extraction method mechanism can operate in any simple container and at low temperature. The low temperature is benefits to extraction process that involved thermolabile compounds; compounds that sensitive when high heat subjected. This method made it is the most cheap and simple operation extraction method since do not require any expensive specific device to operate.

In contrast, the disadvantages drawn by this method is low extraction yield, time consuming, require filtration and need large amount solvent used. These methods are fully dependent on contacting of solvent with surface materials which means need an efficient solvent for extraction process. Choices of suitable solvent with large amount needed might increase a cost in production scale. Moreover, longer extraction times in ME also can cause in decreasing of yield of target compound as claimed by Aspe and Fernandez (2011).

2.6.2 Soxhlet Extraction (SE)

2.6.2.1 Principles and Mechanisms

Soxhlet extraction is one of the oldest method and common widely used approaches for conventional extraction of solid constituents. This type of extraction is running by using Soxhlet extractor which is a type of laboratory glassware invented in 1879 by Franz von Soxhlet. It was initially designed for the extraction of a lipid from a solid material and this technique is based on the choice of solvent coupled with the use of heat (Wang and Weller, 2006). However, a Soxhlet extractor is not limited to the extraction of lipids. Basically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent and the impurity is insoluble in the solvent. Soxhlet extraction is a general and well-established technique which surpasses in performance other conventional extraction techniques except for in limited field of applications; the extraction of thermolabile compounds (Luque de Castro and Garcia-Ayuso, 1998). The practical issue that commonly related to Soxhlet is solvent choice, matrix characteristics and operating conditions.

There are five main components in the Soxhlet extractor. The components are condenser, thimble, extraction chamber, siphon arm and round boiling flask. The grinded plant material is placed in a thimble-holder and filled with solvent from a distillation flask which is then extracted using a solvent via the reflux cycle (Lau *et al.,* 2010). In a reflux cycle process, the solvent is evaporated due to operating temperature at the boiling point of the solvent. As the solvent evaporate, the solvent hits the condenser resulting in condensation of the solvent due to temperature

differences. When the liquid reaches the overflow level, a siphon functions to aspirates the solution of the thimble-holder and unloads it back into the distillation flask carrying extracted solutes from plant material into the solvent (Luque de Castro and Garcia-Ayuso, 1998). Solute is left in the flask and fresh solvent passes back into the plant solid bed in the thimble as the solvent is evaporated. The operation is repeated until complete extraction is achieved. The non-soluble of the extracted solid remains in the thimble and it is usually discarded after extraction process. Once extraction process is complete, the solvent is removed from sample by rotary evaporator which yielding the extracted compound.

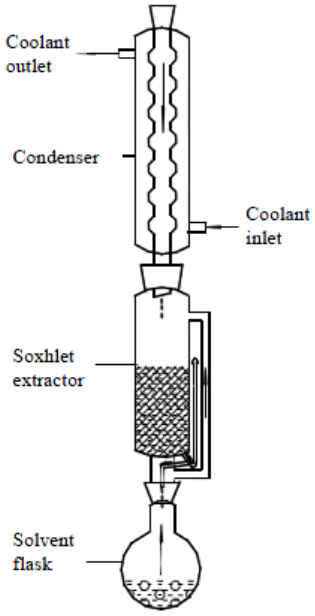

Figure 2.7 Soxhlet extractor (Wang and Weller, 2006)

2.6.2.2 Advantages and Disadvantages of Soxhlet extraction

The outstanding advantages of the Soxhlet extraction are repeatedly brought of sample into contact with the fresh solvent which helping to displace transfer equilibrium. Besides that, the temperature of the system remains relatively high since the heat applied to the distillation flask reaches the extraction cavity and no filtration requirement after leaching process (Luque de Castro and Garcia-Ayuso, 1998). According to Chan *et al.,* (2008), the application of heat in Soxhlet facilitates the cell rupture and leaching which thereby improve the mass transfer of antioxidant compound from the plant matrix into the solvent.

Meanwhile the drawbacks of this system are long extraction time requirement and large amount of solvent used (Wang *et al.,* 2010; Min and Chun, 2005). Plus, Soxhlet also depending in efficiency of solvent in increasing the extraction yield since agitation cannot be provided to accelerate the process. Furthermore, the possibility of thermal decomposition of the target compounds cannot be neglected as the extraction occurs at the boiling point of the solvent for a long time (Luque de Castro and Garcia-Ayuso, 1998).

2.6.3 Ultrasonic-assisted Extraction (UAE)

2.6.3.1 Principles and Mechanisms

Ultrasonic extraction is an extraction technique that employing sound waves principles. Sound waves which have frequencies higher than 20 kHz are mechanical vibrations in a solid, liquid and gas (Luque de Castro and Garcia-Ayuso, 1998). Sound waves travel in a matter and they cause an expansion and compression of fluid movement which results in the cavitation phenomenon (Lau *et al.*, 2010). Cavitation phenomenon can be described as the formation, growth and collapse of vapour filled in bubbles of the liquid. Through expansion, the molecules are pulling apart and the compression effect pushes them together. The expansion creates bubbles in a liquid and produce negative pressure that causes the swelling of the cells and the breakdown of cell walls. Due to limited "space" for the bubbles to enlarge, the bubbles collapse asymmetrically in the container causes impingement by micro-jets resulting in particle breakdown (Shirsath *et al.*, 2012). Breakdown of particle developed the cracks in the cell wall which increases permeability of plant tissues in assisting the penetration of the solvent into the plant matrix as well as washing out of the extracts. However, beyond a certain limit there is depletion of ultrasonic energy as no further increase can be seen owing to the limitations of equilibrium extraction (Romdhane and Gourdon, 2002). As claimed by Vinatoru (2001), ultrasonic mechanism allows high diffusion rates and across the cell wall in simple washing out of the cell contents in seconds. In other words, the mechanical effects of ultrasound induce a greater penetration of solvent into cellular materials and improve mass transfer rate. Therefore, efficient cell disruption and effective mass transfer has been

cited as two major factors of enhancement of extraction with ultrasonic-assisted extraction techniques (Mason *et al.*, 1996). Meanwhile, the common practical issue for ultrasonic is such as moisture content of plant material, particle size, solvent used, frequency, temperature and time should be considered.

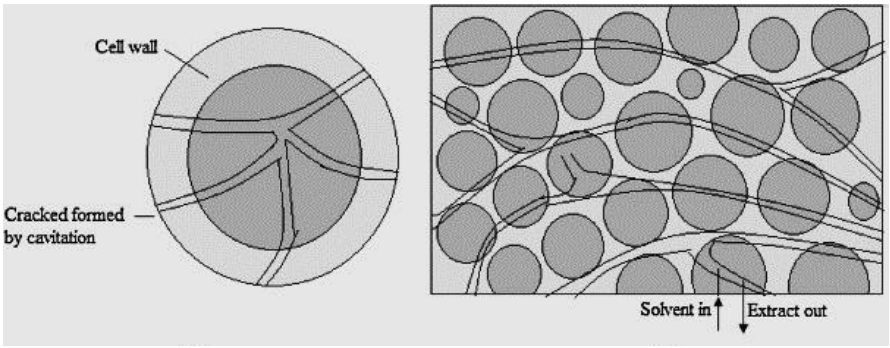

Figure 2.8 Ultrasonic mechanisms (Shirsath *et al.*, 2012)

2.6.3.2 Advantages and Disadvantages of Ultrasonic-assisted extraction

The main advantage of ultrasonic-assisted extraction in solid-liquid extraction is it can accelerate extracting process and improve extraction yield of bioactive compound with faster kinetics (Dong *et al.,* 2010). This advantage can be seen through studies by Li *et al.,* (2004) where yield of oil extracted from soybeans by using ultrasonic-assisted extraction significantly increase. Study by Toma *et al.,* (2001); Xia *et al.,* (2012) stated that collapse of cavitation bubbles near plant tissue in ultrasonic surfaces creates microjets, resulting in tissue distruption and a good solvent penetration into the tissue matrix in shorter time compare to any conventional method. In essence, ultrasonic can decrease the operating temperature which allowing the extraction of thermolabile compounds Compared with other novel extraction techniques such as microwave-assisted extraction, the ultrasound apparatus is simple, cheaper and easy handling operation.

Nevertheless, the drawbacks of ultrasonic-assisted extraction such as heat generated during process should be noted. The sonication time also need to be considered as excess sonication time can damage the quality of extracts (Pan *et al.,* 2002). Additionally, ultrasonic requires a medium such as water for radiation of the sound waves. The presence of a dispersed phase contributes to the ultrasound wave attenuation and the active part of ultrasound inside the extractor is restricted to a zone located in the area of the ultrasonic emitter.

2.7 Extraction Solvent

Choices of solvent in any extraction method are crucial in determining the extraction yield. An elementary aspect in the choice of solvent is solvent viscosity, solvent type and their composition. Solvents with lower viscosities tend to increase extraction rate since they can pass through capillary of plant cells easily and faster than any other solvent with higher viscosities. This behaviour was prove in study by Guillen *et al.*, (1991), where 27 organic solvents were compared and the lowest solvent viscosities gave a high extraction yield. Meanwhile, type of solvent used in extraction process also should be noted. The solvent must not react with any bioactive compound of interest and should keep the targeted compound stability (Aytul, 2010). Water, *n*-hexane, methanol, ethanol and acetone are the most widely employed type of solvent used for extraction of bioactive compounds. In other hand, employing solvent mixtures such as acetone-water are environmentally favourable compared to pure solvent. This system was found can enhance the extraction yields by improving the solubility of bioactive compounds thus increase the interaction of the targeted compounds with the extraction solvent (Mustafa and Turner, 2011). Therefore, 70% acetone was chose in this study due to its efficiency in extracting bioactive compound from *C.caudatus* (Nurain, 2012). This is supported by Gallo *et al.*, (2010) that 70% acetone is a good extraction solvent for plant pigments as it breaks down cell walls and it is miscible with the pigment.

2.8 DPPH Radical Scavenging Assay

Many different analytical methods are employed in screening tests to evaluate the effectiveness of antioxidant compound of a plant samples. Antioxidant effectiveness is measured by monitoring the inhibition of oxidation of substrate using instrumental method. 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging assay is one of the most common applied assay method instead of Superoxide Dismutase (SOD) activity assay and Ferric reducing antioxidant potential (FRAP) assay in measuring antioxidant activity. This assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical. Stable free radical DPPH is reduced corresponding hydrazine when it reacts with hydrogen donors from antioxidant compound (Sanchez-Moreno, 2002). DPPH has a deep violet colour in ethanol or methanol solution and turn yellowish when being neutralized. According to Kalia *et al.,* (2008), change of DPPH colour is due to presence of antioxidant compound which can donate hydrogen or electron reacts with radical DPPH thus reducing the DPPH radicals into stable molecules. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 515–528 nm wavelength (William *et al.,* 1995). Sanchez-Moreno (2002) also state that DPPH assay was considered as a valid and easy assay for evaluating scavenging activity of antioxidant compound since the DPPH radical compound is stable radical and does not have to be generated as in other radical scavenging assays.

2.9 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is mostly utilized method for determination of bioactive compounds. In HPLC, there are special pumps which can deliver constant mobile phase flow rates through the column. The sample containing molecules to be separated is injected into the flowing mobile phase just before it enters the column. In a chromatographic separation process, the concentration of the individual components in the effluent stream from the column needs to be monitored. A plot of the concentration of different components as function of time or cumulative effluent volume is called chromatogram (Ghosh, 2006, p.156). The concentration of the sample can be calculated by the area of peak based on the retention time of standard.

2.10 Rotary Evaporator

A rotary evaporator or commonly called Rotavap is equipment that being used either in laboratory scale or plant scales for removal of solvents from mixture of sample-solvent samples by evaporation. A simple rotary evaporator system was invented by Lyman in 1950. It was then first commercialized by the Swiss company Büchi in 1957, and patented in 1964. Other rotary evaporator manufacturers include Heidolph, Yamato, IKA, Stuart and EYELA. The most common form is the bench-top unit, though large scale (20 to 50 L) versions are used in pilot plants in commercial chemical operations.

There are seven main components of a modern rotary evaporator which are motor, vapor duct, vacuum system, heated fluid bath, condenser, condensate collecting flask and mechanical or motorized mechanism. The description of each components are as follows; motor is a unit which rotates the evaporation flask or vial containing sample, a vapor duct acts as the axis for sample rotation and as vacuum-tight conduit for the vapor being drawn from of the sample, a vacuum system is to substantially reduce the pressure within evaporator system, a heated fluid bath function to heat the sample, a condenser is place where the coolant passes, condensate collecting flask at the bottom of the condenser is to catch the distilling solvent and finally, a motorized mechanism is to lift the evaporation flask from the heating bath. Rotary evaporator is functioning by lowering the pressure above a bulk liquid thus lowers the boiling points of the solvent in it which is the one that need to be removing from a mixture of sample-solvent after an extraction process. Rotary evaporation is most often and conveniently applied to separate "low boiling" solvents such as *n*-hexane or ethyl acetate from compounds (Lee, 2009).

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter illustrates the extraction process based on different extraction method set which are maceration extraction, Soxhlet extraction and ultrasonic-assisted extraction. The subchapter covers in this chapter was extraction preparation, extraction procedure, separation procedure and analysis procedure. These methodologies were being used thoroughly in this study.

3.2 Materials Used

3.2.1 Plant Materials

C.caudatus or known by its local name “ulam raja” was selected in this study. The plants were taken freshly in Kampung Pandan, Kuantan, Pahang and kept in chiller at 4°C until further experiment. Figure 3.1 shows purchased *C.caudatus.*

Figure 3.1 *Cosmos caudatus*

3.2.2 Chemicals and Reagent

All the chemicals and reagents used in this study were analytical grade. Acetone (99.98%), ascorbic acid (Vitamin C), 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH), potassium dihydrogen phosphate (KH_2PO_4), and methanol HPLC grade were purchased from Sigma-Aldrich Chemicals. The physical and chemical properties of chemicals used are attached as in Appendix A.1.

3.3 Extraction Preparation

3.3.1 Sample Preparation

The fresh *C.caudatus* was cleaned first using deionized water to remove any debris. Then, the leaves was isolated from its stems and dried in Memmert oven as shown in Figure 3.2 at 42 °C for 24 hour (Shui *et al.*, 2005). After that, the sample was grounded to fine powder with a blender to provide larger surface area for better contact between plant matrix and the solvent. The dried powder form of sample then was transferred into an amber bottle to prevent direct light exposure and stored in chiller at 4°C until further study (Wong *et al.*, 2006).

Figure 3.2 Drying oven (Brand: Memmert)

3.3.2 Solvent Preparation

Solvent used for all extraction procedure were distilled water as a control and 70% acetone. 70% acetone was prepared by diluting acetone with distilled water. The formula used as in Equation 3.1.

$$M_1V_1 = M_2V_2 \qquad \text{(Eq. 3.1)}$$

Where,

M_1= Initial concentration of solvent

M_2= Final concentration of solvent

V_1= Volume need from initial concentration of solvent

V_2= Final volume needed

3.4 Extraction Procedure

3.4.1 Extraction of Antioxidant Compound by Maceration Extraction (ME)

Ten gram of sample was weighted. Next, the sample and 200 ml of 70% acetone was poured into the conical flask. The conical flask was covered with aluminum foil to prevent direct light exposure. Then, the mixture was placed on a shaker CERTOMAT *SII* as shown in Figure 3.3 and was run at 8, 16, 24 hours with agitation at room temperature (Biesaga, 2011). After extraction, the crude extracts were filter through filter paper and centrifuge at 4000 rpm for 10 minutes at 4°C (Jun *et al.,* 2011). Subsequently, the supernatant which was the mixture sample-solvent was transferred into an amber bottle and stored in chiller until further experiment procedure. The procedure was repeated by using 200 ml of distilled water as a solvent.

Figure 3.3 Orbital shaker (Brand: CERTOMAT *SII*)

3.4.2 Extraction of Antioxidant Compound by Soxhlet Extraction (SE)

Ten gram of sample was weighted and put into a thimble of Soxhlet extractor as shown in Figure 3.4. Then, the thimble was inserted into Soxhlet chamber. Two hundred ml of 70% acetone was poured into round bottom flask of Soxhlet extractor. After that, the extractions were run for 4, 8, 12 hours at boiling point of the solvent. The extraction process was start once the heating is begun to heat the solvent with the constant temperature. The crude extract was collected and processed as in ME procedure. The procedure was repeated by using 200 ml of distilled water as a solvent.

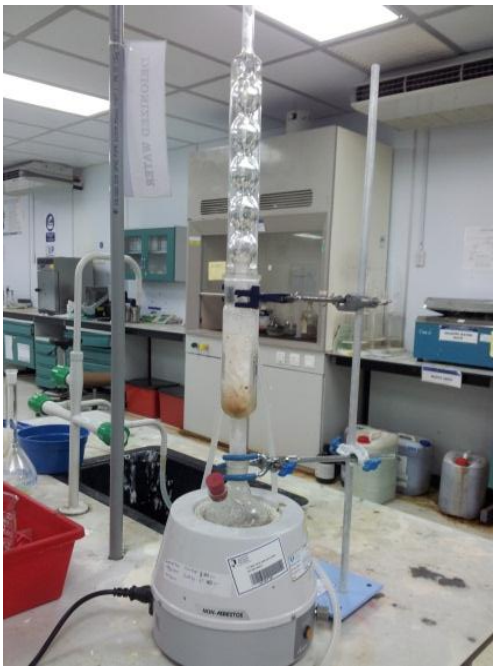

Figure 3.4 Soxhlet extractor (Brand: Favorit)

3.4.3 Extraction of Antioxidant Compound by Ultrasonic-assisted Extraction (UAE)

Ten gram of samples and 200 ml of 70% acetone were inserted into conical flask. The sample and the solvent was mix well to ensure that all the samples were immersed in the solvent. Then, the top of the conical flask was covered with aluminum foil to prevent loss of solvent volume during extraction process and placed in the ultrasonic bath. Extraction were performed using CREST ultrasonic bath as shown in Figure 3.5 at temperature 35 °C with sonication time at 30, 60, and 90 minutes (Wang *et al.,* 2010). The crude extracts obtained were filtered and processed as done previously in ME procedure. The procedure was repeated by using 200 ml of distilled water as a solvent.

Figure 3.5 Ultrasonic extractor (Brand: CREST)

3.5 Separation Procedure

The supernatant or mixture sample-solvent saved as described from previous section was separated using rotary evaporator Heidolph LABOROTA 4000 as shown in Figure 3.6. The mixture sample-solvent were first transferred into receiving flask of the rotary evaporator and was concentrated at boiling point temperature of the solvent with rotary speed of 150 rpm. Excess solvent was vaporizing due to the differences in boiling point with the sample. Recovery solvent was collected in the collecting flask while the purify sample remained in the receiving flask. The purify sample then was stored in an amber bottle and stored in chiller for further experiment procedure.

Figure 3.6 Rotary evaporator (Brand: Heidolph)

3.6 Analysis Procedure

3.6.1 Determination of antioxidant compound using DPPH assay

The radical scavenging activity of *C. caudatus* extracts was determined using the Hitachi UV-VIS spectrophotometer as shown in Figure 3.7. First, stock solution was prepared by dissolving 0.025 g of 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) with 100 ml methanol and kept in an amber bottle. After that, for DPPH working solution, a 1:10 dilution of stock was prepared with methanol. Then, 3.9 ml of DPPH working solution was mixed with 0.1 ml extract solution. The mixture solution was shaken vigorously with vortex and incubated in dark place for 30 minutes at room temperatures. The reaction solution absorbance was measured at 517 nm after 30 min incubation (Margitanova *et al.,* 2012). While for the blank solution, methanol was used. The radical scavenging activity was calculated as a percentage of DPPH scavenging activity using the Equation 3.3. Where $A_{control}$ is the absorbance of DPPH working solution and A_{sample} is the absorbance of the DPPH working solution with an extracts solution added.

$$\text{Scavenging activity } (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \qquad \text{(Eq 3.2)}$$

Figure 3.7 Uv-vis spectrophotometer (Brand: Hitachi)

3.5.3 Determination of Ascorbic Acid Content Using High Performance Liquid Chromatography (HPLC)

All ascorbic acid existence and ascorbic acid contents analysis in *C. caudatus* extract was performed by using AKTAexplorer HPLC method (Lim *et al.*, 2006). HPLC used in this study was shown in Figure 3.8. In HPLC method analysis; C-18 column (3.9 x150 mm, 5 mm particle size), mobile phase 0.05 M potassium dihydrogen phosphate and 0.05 M solution of potassium dihydrogen phosphate-methanol (70:30), flow rate 1.0 mL/min and 254 nm detection wavelength were used (Pan *et al.*, 2002).

3.5.3.1 HPLC Mobile Phase Preparation

The 0.05 M potassium dihydrogen phosphate (KH_2PO_4) mobile phase was prepared by weighted 6.805 g of potassium dihydrogen phosphate powder and dissolved into 1 L of ultrapure water. The solution was then filtered through 0.45µm membrane filter and sonicated about 30 minutes in ultrasonic to remove the bubbles. Five hundred ml of the 0.05 M potassium dihydrogen phosphate mobile phase was used in HPLC.

Next, for the preparation of second mobile phase which is 0.05 M solution of potassium dihydrogen phosphate-methanol (70:30), the potassium dihydrogen phosphate was weighted and dissolved in 1 L of ultrapure water. Afterwards, 700 ml of this 100% potassium dihydrogen phosphate mobile phase solution was diluted with 300 ml of methanol HPLC grade. The solution later was processed as previously done in first mobile phase preparation.

3.5.3.2 HPLC Standard Curve Preparation

For the standard solution of HPLC analysis, standard solution of ascorbic acid was prepared. The stock solution of ascorbic acid was prepared at concentration 10 000 ppm where 1 g of ascorbic acid was dissolved into 100 ml of ultrapure water. Next, the other concentration of standard solution of ascorbic acid was prepared at 100 ppm, 200 ppm, 400 ppm, 600 ppm, 800 ppm and 1000 ppm by diluting the stock solution of ascorbic acid with ultrapure water to give respective set standard solution of ascorbic acid concentration. All the standard solutions were filtered through 0.45 µm membrane filter and 10µl of each standard was injected into HPLC.

3.5.3.3 HPLC Sample Preparation

The extracts sample for HPLC analysis was filtered through 0.45 µm syringe filter unit using sterile syringe and inserted into labelled vials. The sterile syringe was used to minimize contamination for better analysis results. About 10 µl of sample was injected into HPLC (Pan *et al.*, 2002). The presence of ascorbic acid can be noticed by the appearance of peak at certain range of time. The amount of ascorbic acid content was calculated from the area of the peak.

Figure 3.8 High Performance Liquid Chromatography (Brand: Agilent)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter discusses the outcomes of this study that related to the objectives and scopes. The topic cover in this chapter is ascorbic acid content, antioxidant activity and HPLC analysis of *Cosmos caudatus* based on all the parameters set which are the extraction method (maceration extraction, Soxhlet extraction and ultrasonic-assisted extraction) and extraction time. The HPLC analysis was done based on highest ascorbic acid content from each extraction method with the highest antioxidant activity. Distilled water was used in this study as a control in each extraction method.

4.2 Results and Discussion

4.2.1 Ascorbic Acid Content Analysis from *Cosmos caudatus* Extract

Ascorbic content analysis is important in this study in determining the efficiency each extraction method on recovery of ascorbic acid content from *C.caudatus*. Ascorbic acid is one of the antioxidant compounds that have great importance in biochemical reactions as a reducing agent. In the ascorbic acid content analysis, the standard curve ascorbic acid was plotted in order to determine the concentration of *C.caudatus* extracts. The concentrations ascorbic acid were set at 400, 800, 1200, 1600 and 2000 mg/L by diluted ascorbic acid using distilled water. Each of the ascorbic acid concentration absorbance then was monitored at 517 nm using Uv-vis spectrophotometer. Table 4.1 shows tabulated data for ascorbic acid standard curve.

Table 4.1 Data for ascorbic acid standard curve

Ascorbic acid concentration (mg/L)	**Absorbance unit**
0	0
400	0.07
800	0.132
1200	0.181
1600	0.258
2000	0.325

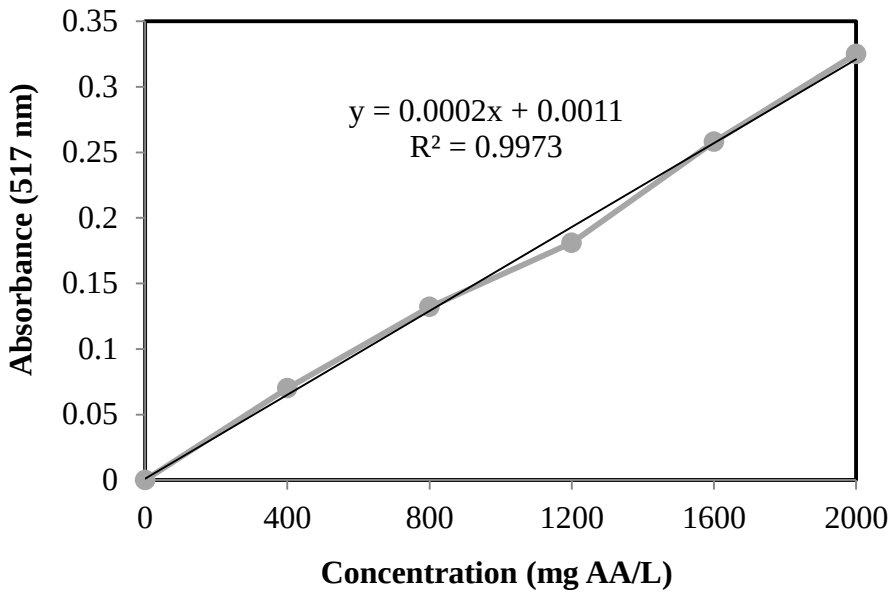

Figure 4.1 Ascorbic acid standard curve

Based on Figure 4.1 the concentration of 2000 mg/L demonstrate the highest absorbance unit with 0.325 followed by the concentration of 1600 mg/L, 1200 mg/L, 800 mg/L and 400 mg/L with the values of absorbance units 0.258, 0.181, 0.132 and 0.07 respectively. The relationship between the absorbance unit and concentration of ascorbic acid was directly proportional with R-squared value of 0.9973. The amount of ascorbic acid content (AAC) in *C.caudatus* extracts was determined using equation 4.1 and expressed in mg ascorbic acid equivalent per 1 g sample (mg AAE/g).

$$\text{AAC} = \frac{\text{Sample concentration } \left(\frac{\text{mg AA}}{\text{L}}\right) \times \text{Solvent volume (L)}}{\text{Mass sample (g)}} \qquad \text{(eq. 4.1)}$$

4.2.1.1 Effect of Maceration Extraction (ME) on Ascorbic Acid Content

Table 4.2 shows ascorbic acid content of *C. caudatus* extracted using maceration extraction. The ascorbic acid content in each extracts was measured using equation 4.1 in section 4.2.1. Based on Table 4.2, different solvents used shows different values of ascorbic acid contents. *C. caudatus* was extracted using two different polarities solvent system which is distilled water and 70% acetone (v/v). While in term of time, the extraction was performed at three different time; 8, 16 and 24 hour. Each extraction time also shows significant difference value of ascorbic acid content in both type solvent used. All the data obtained was plotted in Figure 4.2.

Table 4.2 Data analysis of the *C.caudatus* extracts by maceration extraction

Time of Extraction (hour)	**Ascorbic Acid Content (mg AAE/g)**	
	Distilled Water	**70% Acetone**
8	1.857	2.857
16	2.790	3.757
24	4.390	5.190

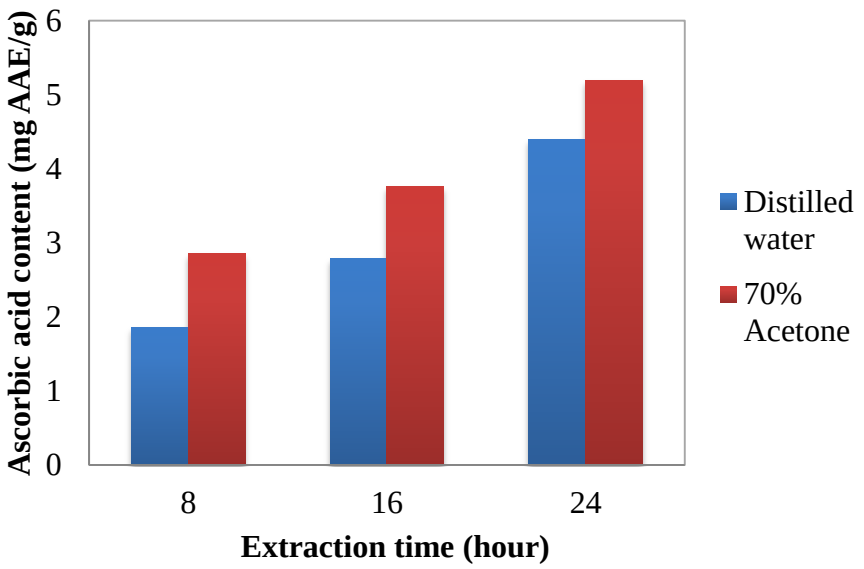

Figure 4.2 Effect of ME time on ascorbic acid content (solvent: 70% (v/v) of 200 ml acetone, *C. caudatus*: 10.0 g, solid to liquid ratio: 1/20 (w/v), room temperature: about 25 °C, agitation speed: 100 rpm)

Figure 4.2 demonstrates the effect of ME time on ascorbic acid contents using both distilled water and 70% acetone as solvent. By taking the extraction process using 70% acetone as the solvent, the highest ascorbic acid content was exhibited by 24 hour extraction time with 5.190 mg AAE/g. This is followed by 16 hour and 8 hour extraction time with 3.757 mg AAE/g and 2.857 mg AAE/g respectively. This result indicates that the ascorbic acid content increased with the increase of extraction time. The extraction efficiency of bioactive compounds in ME is strongly affected by extraction time. According to Lau *et al.,* (2010), longer extraction time extend diffusion time and facilitate solubilization through penetration of solvent into plant matrix. This pattern was similarly seen in previous study by Aspe and Fernandez (2011) in extracting *Pinus* radiata Bark using maceration extraction where increasing yield obviously seen as extraction times increased.

Meanwhile, in terms of solvent used, 70% acetone extracts exhibit highest value of ascorbic acid content compared to distilled water extracts in all extraction time. Considering the highest amount ascorbic acid content from both solvent used, 70% acetone yield up to 3.757 mg AAE/g while distilled water extracts could only yield 2.790 mg AAE/g of ascorbic acid. This is due to low viscosity of 70% acetone than distilled water. Aytul (2010) in his study states that solvents with lower viscosities increase extraction yield since they can pass through plant matrix faster than any other solvent with higher viscosities. These finding revealed that 24 hour extraction time and 70% acetone is an efficient time and solvent system in extraction of ascorbic acid from *C.caudatus* by ME method respectively.

4.2.1.2 Effect of Soxhlet Extraction (SE) on Ascorbic Acid Content

Table 4.3 shows ascorbic acid content of *C. caudatus* extracted using Soxhlet extraction method. The ascorbic acid content in each extracts were measured and calculated as previously described in section 4.2.1. Based on Table 4.3, the same pattern of solvent system on ascorbic acid content was drawn by SE where 70% acetone extracts have high ascorbic acid content than distilled water extracts. SE method was performed at three different time; 4, 8 and 12 hour. Difference extraction time yield difference ascorbic acid content. All the data obtained was plotted in Figure 4.3.

Table 4.3 Data analysis of the *C.caudatus* extracts by Soxhlet extraction

Time of Extraction (hour)	**Ascorbic Acid Content (mg AAE/g)**	
	Distilled Water	**70% Acetone**
4	6.823	9.290
8	8.523	13.257
12	7.090	10.423

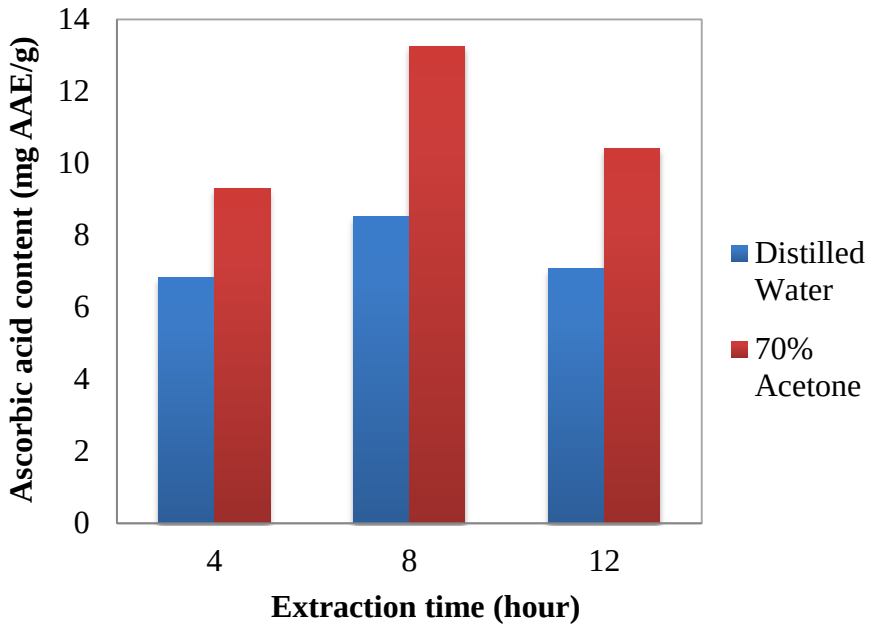

Figure 4.3 Effect of SE time on ascorbic acid content (solvent: 70% (v/v) of 200 ml acetone, *C. caudatus*: 10.0 g, solid to liquid ratio: 1/20 (w/v), temperature: 56 °C)

The result of Figure 4.3 illustrates the effect of SE time on ascorbic acid contents using both distilled water and 70% acetone as solvent. From the graph, the ascorbic acid content significantly increased after 4 hour (9.290 mg AAE/g) and reached the highest after 8 hour (13.257 mg AAE/g) extraction time when 70% acetone was used as solvent. This may due to application of heat in SE that facilitates the cell rupture and leaching which thereby improve the mass transfer of antioxidant compound from the plant matrix into solvent (Chan *et al.*, 2008). Soxhlet works at boiling point of the solvent due to its mechanism that necessary to boil the solvent. Nevertheless, as the extraction time was extended to 12 hour, the ascorbic acid content was decreased to 10.423 mg AAE/g. The decreasing in value of ascorbic acid after 12 hour extraction time was believed due to partly to the decomposition of ascorbic acid at high temperature. This is supported in literature by Wang and Weller (2006) which state that the possibility of thermal decomposition of the target

compounds by SE might occurs due to its extraction mechanism at boiling point of the solvent for a long time. The similar pattern also experienced in previous study by Pan *et al.,* (2002) where at certain Soxhlet extraction time, the yield of bioactive compounds started to decrease after reached its highest yield.

In other hand, the similar pattern in term of effect of solvent were also obtained as in ME method where 70% acetone extracts exhibit highest value of ascorbic acid content against distilled water extracts in all extraction time. The reason behind efficiency 70% acetone in SE method was likewise state as in previous section which due to low viscosity. This is agreed by Gallo *et al.,* (2010) that 70% acetone is a good extraction solvent for plant pigments as it breaks down cell walls and it is miscible with the pigment. For that reason, it can be concluded that for SE method, the higher ascorbic acid content can be achieved at 8 hour extraction time with 70% acetone as solvent system from *C. caudatus*.

4.2.1.3 Effect of Ultrasonic-assisted Extraction (UAE) on Ascorbic Acid Content

Table 4.3 demonstrates ascorbic acid content of *C. caudatus* extracted using ultrasonic-assisted extraction. The ascorbic acid content in each extracts was determined as calculated in section 4.2.1. Data in Table 4.4 shows that 70% acetone extracts have highest ascorbic acid contents than distilled extracts by UAE method in all extraction time set. This trend was similar as seen in both ME and SE method. In UAE method, three different extraction times were set; 30, 60, and 90 minutes. All three extraction times exhibit different ascorbic acid contents. All data in Table 4.4 were expressed in bar chart graph in Figure 4.4.

Table 4.4 Data analysis of the *C.caudatus* extracts by ultrasonic-assisted extraction

Time of Extraction (minute)	**Ascorbic Acid Content (mg AAE/g)**	
	Distilled Water	**70% Acetone**
30	13.357	17.023
60	16.557	26.590
90	11.890	22.657

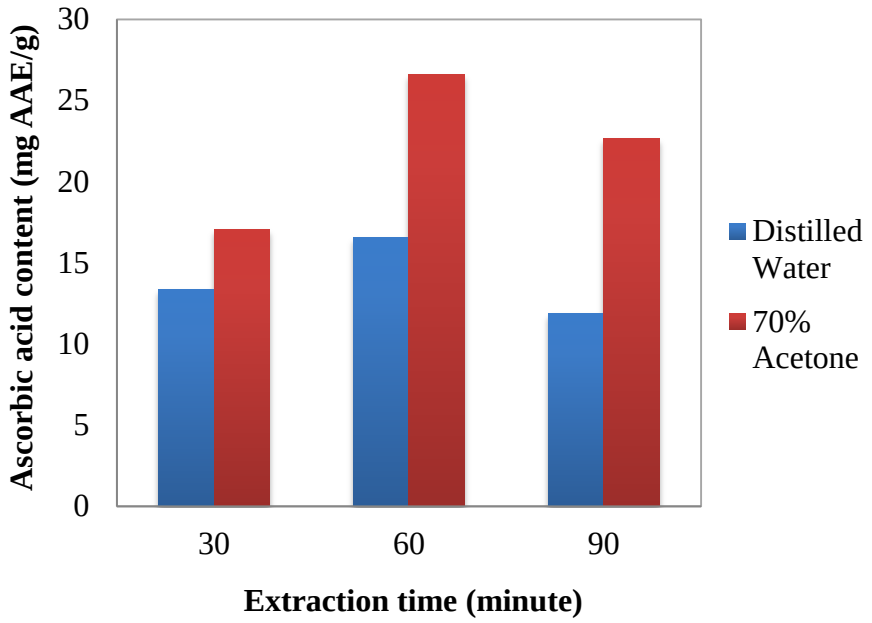

Figure 4.4 Effect of UAE time on ascorbic acid content (solvent: 70% (v/v) of 200 ml acetone, *C. caudatus*: 10.0 g, solid to liquid ratio: 1/20 (w/v), temperature: 35 °C, power: 240 W, frequency: 45 kHz)

Influence of time on the extraction of ascorbic acid by UAE method is shown in Figure 4.4. The results indicate that the ascorbic acid content significantly increased with time. Taking the 70% acetone extraction process first, where in 30 minute extraction time, the amount ascorbic acid content was 17.023 mg AAE/g. This is followed by 60 minute extraction time where highest ascorbic acid content was reached (26.560 mg AAE/g). The highest ascorbic acid content released is due to UAE system itself that accelerate the release of targeted compounds; ascorbic acid. Ultrasonic principle is based on cavitation effects, which increase mass transfer between the solvent and the plant tissue. This collapse of cavitation bubbles near plant tissue surfaces creates microjets, resulting in tissue distruption and a good solvent penetration into the tissue matrix (Toma *et al.*, 2001). Conversely, a slightly decrease of ascorbic acid content was observed at 90 minute extraction time with 22.657 mg AAE/g which can be due to overexposure to the radiation or thermal

degradation. Thermal degradation might occur as the irradiation time increase which creates heat that can cause degradation towards bioactive compound (Pan *et al.*, 2002). Similar behaviour was observed in the extraction of flavonoids from cultured cells of *Saussurea medusa* Maxim (Gao and Liu, 2005).

Meanwhile, a big difference of ascorbic acid content was clearly seen between distilled water extracts and 70% acetone extracts. Using distilled water as extraction solvent in UAE method were least effective (16.557 mg AAE/g) compared to 70% acetone (26.590 mg AAE/g) in all extraction time. In particular, the similar trend was drawn by UAE method as in ME and SE method where 70% acetone extracts exhibit higher ascorbic acid content than distilled water. Thus, it can be said that employing 60 minute extraction time and 70% acetone in UAE method shows a high ascorbic acid content.

4.2.1.4 Comparison of Different Extraction Methods on Ascorbic Acid Content

A number of extraction methods, including maceration extraction (ME), Soxhlet extraction (SE) and ultrasonic-assisted extraction (UAE) were employed on recovery of ascorbic acid content from *C. caudatus*. In this section, the comparison between these three extraction methods was discussed. All comparison was made based on the highest ascorbic acid content from previous section where ME; 24 hour extraction time, SE; 8 hour extraction time and UAE; 60 minute extraction time. Table 4.5 shows a summarize data of highest ascorbic acid content for each extraction method and plotted in Figure 4.5.

Table 4.5 Data analysis of the *C.caudatus* extracts by different extraction method

Extraction Method	**Ascorbic Acid Content (mg AAE/g)**
Maceration Extraction (ME)	5.190
Soxhlet Extraction (SE)	13.257
Ultrasonic-assisted Extraction (UAE)	26.590

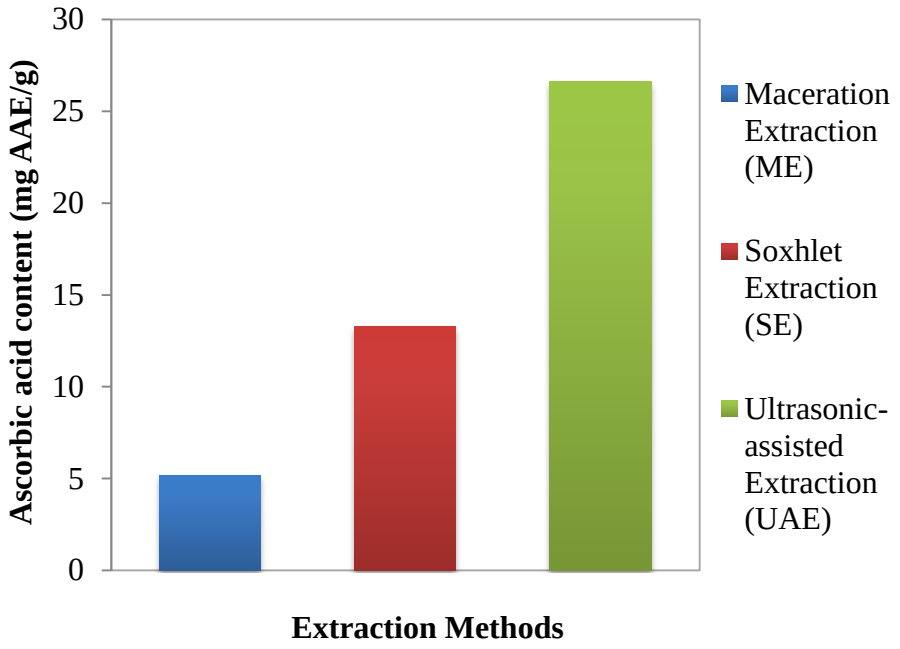

Figure 4.5 Comparison of different extraction method on ascorbic acid content (solvent: 70% (v/v) of 200 ml acetone, *C. caudatus*: 10.0 g, solid to liquid ratio: 1/20 (w/v), extraction time: ME: 24 hour, SE: 8 hour, UAE: 60 min)

Figure 4.5 displays the ascorbic acid content was reached after 24 hour (5.190 mg AAE/g), 8 hour (13.527 mg AAE/g) and 60 minute (26.590 mg AAE/g) when maceration extraction, Soxhlet extraction and ultrasonic-assisted extraction method were used respectively. The lowest ascorbic acid content by ME was understood due to its mechanism that only depends on contacting of solvent with surface of plant matrix without any inducement factor. According to Gao and Liu (2005), ME is easy to carry out but have disadvantages in terms of lowest extraction efficiency. Similar behaviour was experienced by Aspe and Fernandez (2011) where ME extracting yield only range between 2% to 25%. Meanwhile, an increase of ascorbic acid content was seen by SE method. This is because SE always brought the sample into contact with fresh portions of the solvent and heat subjected during extraction process. The heat applied contributes to increase process efficiency since the saturation equilibrium constant is displaced, favoring extraction (Aspe and

Fernandez, 2011). Though, still SE method had very low extraction efficiency of ascorbic acid because of the long extraction time factor. Both ME and SE are time consuming process based on mixing and heat to increase the mass transfer rate.

In contrast, UAE method are physical field enhanced methods and greatly increase improved the extraction efficiency of ascorbic acid in comparison with two extraction method mentioned above. UAE mainly depends on the ultrasonic effects of acoustic cavitation but other effects like diffusion and concussion also help in enhancing the extraction process (Hromadkov and Ebringerov, 2003). Additionally, the vibrated and accelerated solid and liquid particles during UAE result the solute quickly diffuses from solid phase to solvent. Thereby, by comparing all the three method (ME, SE and UAE), UAE showed evident advantages with strong penetration forces, shorter extraction time and high efficiency of ascorbic acid content among all extraction methods used hence the most promising and efficient method in extracting antioxidant compounds from *C. caudatus*. Akin result also have been reported by other study on comparing the UAE with ME and SE for the purposes of tanshinones from *Salvia miltiorrhiza bunge* (Pan *et al.,* 2002), for extraction from *Pinus radiata* Bark (Aspe and Fernandez, 2011), for extraction of aloe-emodin (Wang *et al.,* 2010) and for extraction of oxymatrine from *Sophora flavescens* (Xia *et al.*, 2012).

4.2.2 Antioxidant Activity (DPPH assay) Analysis

The antioxidant activities in *C. caudatus* extracts by three different extraction methods was determined by DPPH free radical scavenging activities assay. In this section, all comparison was made based on the highest ascorbic acid content from each method where ME; 24 hour extraction time, SE; 8 hour extraction time and UAE; 60 minute extraction time. Referring to the Table 4.6, as the ascorbic acid content is higher, the scavenging activity is lower. This is because presence of antioxidant compounds which can donate hydrogen reacts with DPPH thus reducing the DPPH radicals (Kalia *et al.*, 2008). The change in colour from deep violet to yellow was observed due to reduction of DPPH radical to stable molecule. Data in Table 4.6 were summarized in Figure 4.6.

Table 4.6 Data analysis of the antioxidant activity of *C. caudatus* extracts using DPPH assay

Extraction Method	**Ascorbic Acid Content (mg AAE/g)**	**Scavenging Activities (%)**
Maceration Extraction (ME)	5.190	88.528
Soxhlet Extraction (SE)	13.257	71.068
Ultrasonic-assisted Extraction (UAE)	26.590	42.208

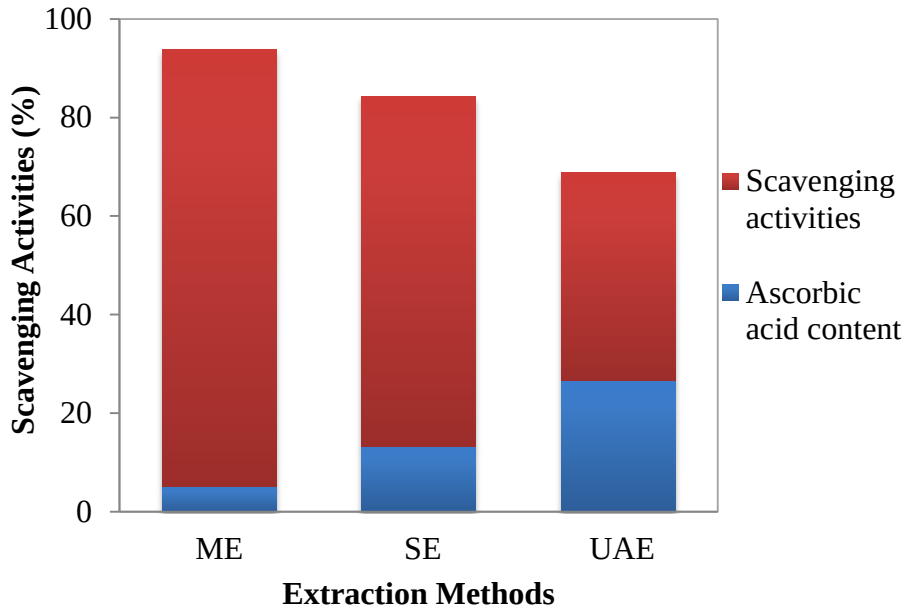

Figure 4.6 Relationship between ascorbic acid content and scavenging activities for each extraction method (solvent: 70% (v/v) of 200 ml acetone, *C. caudatus*: 10.0 g, solid to liquid ratio: 1/20 (w/v), extraction time: ME: 24 hour, SE: 8 hour, UAE: 60 min)

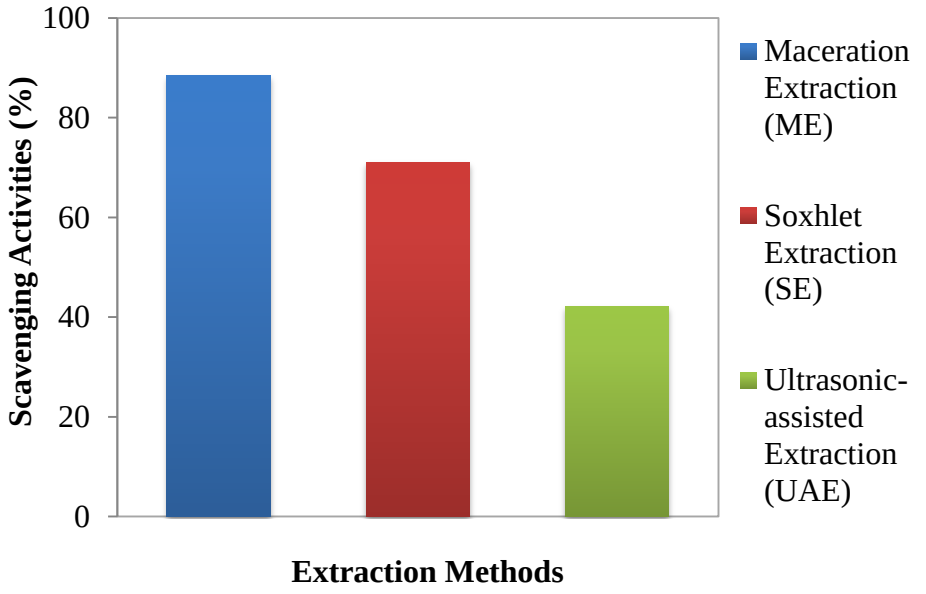

Figure 4.7 Comparison of different extraction method on scavenging activity (solvent: 70% (v/v) of 200 ml acetone, *C. caudatus*: 10.0 g, solid to liquid ratio: 1/20 (w/v), extraction time: ME: 24 hour, SE: 8 hour, UAE: 60 min)

Figure 4.6 illustrates the relationship between ascorbic acid content and antioxidant activity of *C. caudatus* extracts by different extraction method which is ME, SE and UAE. From the graph, higher ascorbic acid content exhibit lower scavenging activities all extraction method employed. This is because higher ascorbic acid content capable in reducing the DPPH radical into stable molecule, thus reducing it scavenging activities (Wong *et al.,* 2006). In other word, antioxidant compound that exhibit lowest scavenging activities shows its efficiency as reducing agents in scavenge free radicals.

Based on Figure 4.7, comparing between the three extraction methods, UAE method extracts exhibit lowest scavenging activities with 42.208% followed by SE and ME with 71.068% and 88.528% respectively. A big difference in scavenging activities value is seen between UAE extracts and two other method extracts (ME and SE) which revealed the efficiency of UAE method. Similar reason on efficiency of UAE extracts is as described in the previous section where due to its mechanism that facilitated cell rupture and penetrating of solvent into plant matrix. Based on this analysis, it is obviously shown that UAE was the most efficient in extracting and preserve antioxidant compound from *C. caudatus*.

4.2.3 High Performance Chromatography Liquid (HPLC) Analysis

4.2.3.1 HPLC Standard Curve

HPLC analysis was made for quantification of ascorbic acid in the *C. caudatus* extracts. Figure 4.8 shows the ascorbic acid standard curve HPLC range that was set from 200, 400, 600 and 800 ppm. The peak area was directly proportional with the amount of ascorbic acid with value of R-squared of 0.99719. The retention time of ascorbic acid was noticed at 2.673 minute.

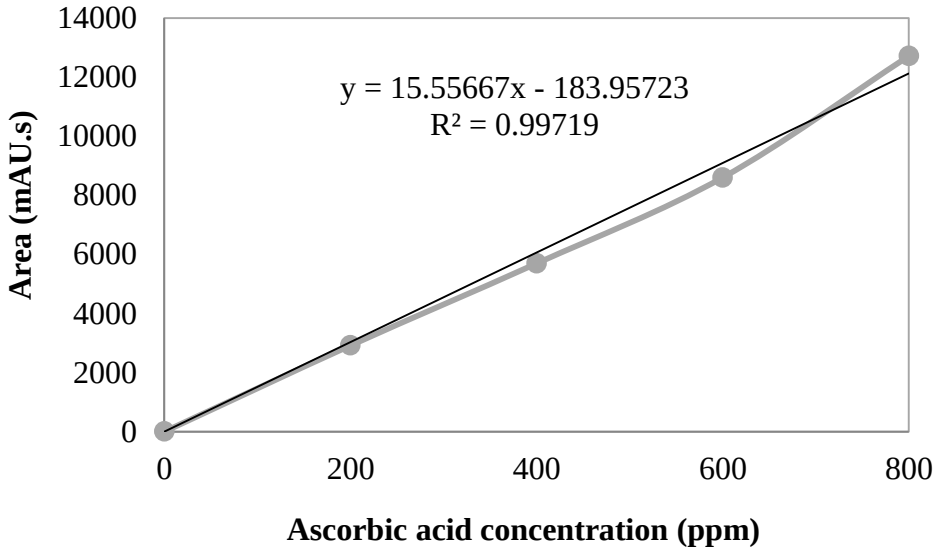

Figure 4.8 Ascorbic acid standard curve (HPLC)

4.2.3.2 Determination of *C. caudatus* Extracts Concentration

Based on the section 4.2.2, the samples which shown high ascorbic acid content from each extraction method was then analyzed using High Performance Chromatography Liquid (HPLC). All the extract concentrations were determined using equation 4.2 and data is shown in Table 4.7.

$$y = 15.55667x - 189.95723 \qquad \text{(Eq. 4.2)}$$

Where;

Y = peak area

m = 15.55667

x = concentration (mg/L)

c = -183.95723

Table 4.7 Data analysis of the antioxidant activity of *C. caudatus* extracts using HPLC analysis

Extraction Methods	**Area Under Peak (mAU.s)**	**Ascorbic Acid Concentration (mg AAE/g)**
Maceration Extraction (ME)	389.716	0.738
Soxhlet Extraction (SE)	569.327	0.968
Ultrasonic-assisted Extraction (UAE)	1712.00	2.440

Based on the results obtained, the concentration of ascorbic acid in *C. caudatus* extracts by UAE method was the highest, followed by SE extracts and finally ME extracts. The trend was similar as in DPPH assay where UAE method shows a high ascorbic acid content than any other method proposed. As in the described in the literature, the efficiency of ultrasonic-assisted extraction in extracting ascorbic acid compound from *C.caudatus* due to expansion and compression mechanism that disrupts cell wall of the plant matrix which later creates a crack that allowing penetration of solvent easily into plant cell in extracting out bioactive compound (Shirsath *et al.,* 2012). In addition, high ascorbic acid content value demonstrate by UAE compare than SE and ME is due to low temperature operating in shorter time which helps in preserve the antioxidant compound from degradation states. This is supported by Pan *et al.,* (2002), where degradation of bioactive compound issue commonly related to high operating temperature and longer extraction times.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, the ascorbic acid content and antioxidant activities are depending on the extraction methods and extraction time. All the three extraction method (maceration extraction, Soxhlet extraction and ultrasonic-assisted extraction) employ in this study exhibit significant value of ascorbic content and antioxidant activity. Based on the results obtained, ultrasonic-assisted extraction extract showed the highest ascorbic acid content with 25.690 mg AAE/g and 42.208% of antioxidant activity after 60 minutes extraction. Meanwhile, Soxhlet extraction exhibit 13.527 mg AAE/g with 71.068% antioxidant activity followed by maceration extraction (5.190 mg AAE/g) with 91.630% antioxidant activity in 8 hour and 24 hour extraction respectively. Moreover, analysis by HPLC also proved that ultrasonic-assisted extraction was an efficient extraction method with 2.440 mg AAE/g followed by Soxhlet extraction and maceration extraction with 0.968 mg AAE/g and 0.738 mg AAE/g accordingly. In other hand, 70% acetone was found as an efficient

solvent system in extracting antioxidant compounds from *C. caudatus* in this study. By considering all the extraction method, ultrasonic-assisted extraction was found to be the most promising and efficient method where highest amount ascorbic acid content was extracted in shorter time. In consequence, antioxidant capacity of *C. caudatus* was greatly influenced by extraction method used.

5.2 Recommendations

For future study, it is recommended that each extraction method should have temperature controller to avoid any irregularities and to get accurate result. Unstable temperature such as in ultrasonic due to its mechanism can cause loss of solvent volume hence affecting the results. The same problems also encountered by Soxhlet where temperature water which acts as cooling agent in condenser was not same in each experiment run and affecting time for solvent to condense. Therefore, cooling circulator with controlled temperature is proposing to overcome this problem. In the future, it is suggested that scanning electron microscope (SEM) observation is made in order to fully understand how each extraction method disrupt plant matrix in extracting out bioactive compounds. Besides that, using other extraction method such as microwave-assisted extraction and supercritical fluid extraction in extraction of *C.caudatus* is suggested for further extraction method comparison. Finally, by looking at ultrasonic-assisted extraction method efficiency in this study, optimization of the ultrasonic-assisted extraction method is proposed for further study to find the optimum condition in extracting bioactive compounds from *C. caudatus*.

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APPENDIX A.1

Physical Properties Solvents used

Table A.1.1 Properties of acetone

Properties	
Molecular formula	C_3H_6O
Molar mass	58.08
Appearance	Colorless liquid
Density	0.791 g/cm3
Melting point	−93 °C, 159 K, -139 °F
Boiling point	57 °C, 330 K, 134 °F
Vapor pressure	24.46 kPa (at 20 °C)
Acidity (pKa)	24.2
Basicity (pKb)	-10.2
Reftractive Index (nD)	1.35900
Viscosity	0.3075 Pa.s (at 20 °C)
Dipole moment	2.91 D
Polarity index	5.1

Table A.1.2 Properties of DPPH

Properties	
Molecular formula	$C_{18}H_{12}N_5O_6$
Molar mass	394.32 g/mol
Appearance	Black to green powder, purple in solution
Density	1.4 g/cm^3
Melting point	135 °C, 408 K, 275 °F (decomposes)
Solubility in water	insoluble

Table A.1.3 Properties of water

Properties	
Molecular formula	H_2O
Molar mass	18.01528(33) g/mol
Appearance	white solid or almost colorless, transparent, with a slight hint of blue, crystalline solid or liquid
Density	1000 kg/m3, liquid (4 °C) (62.4 lb/cu. ft) 917 kg/m3, solid
Melting point	0 °C,(273.15 K)
Boiling point	99.98 °C, 211.97 °F (373.13 K)
Vapor pressure	2.338 kPa (20 °C)
Acidity (p*K*a)	15.74 ~35–36
Basicity (p*K*b)	15.74
Reftractive Index (nD)	1.3330
Viscosity	0.001 Pa.s at 20 °C
Dipole moment	1.85 D
Polarity index	9

Table A.1.4 Properties of ascorbic acid

Properties	
Molecular formula	$C_6H_8O_6$
Molar mass	176.12 g mol^{-1}
Appearance	White or light yellow solid
Density	1.65 g/cm^3
Melting point	190-192 °C, 463-465 K, 374-378 °F (decomp.)
Solubility in water	330 g/L
Solubility in ethanol	20 g/L
Solubility in glycerol	10 g/L
Solubility in propylene glycol	50 g/L
Acidity (pK_a)	4.10

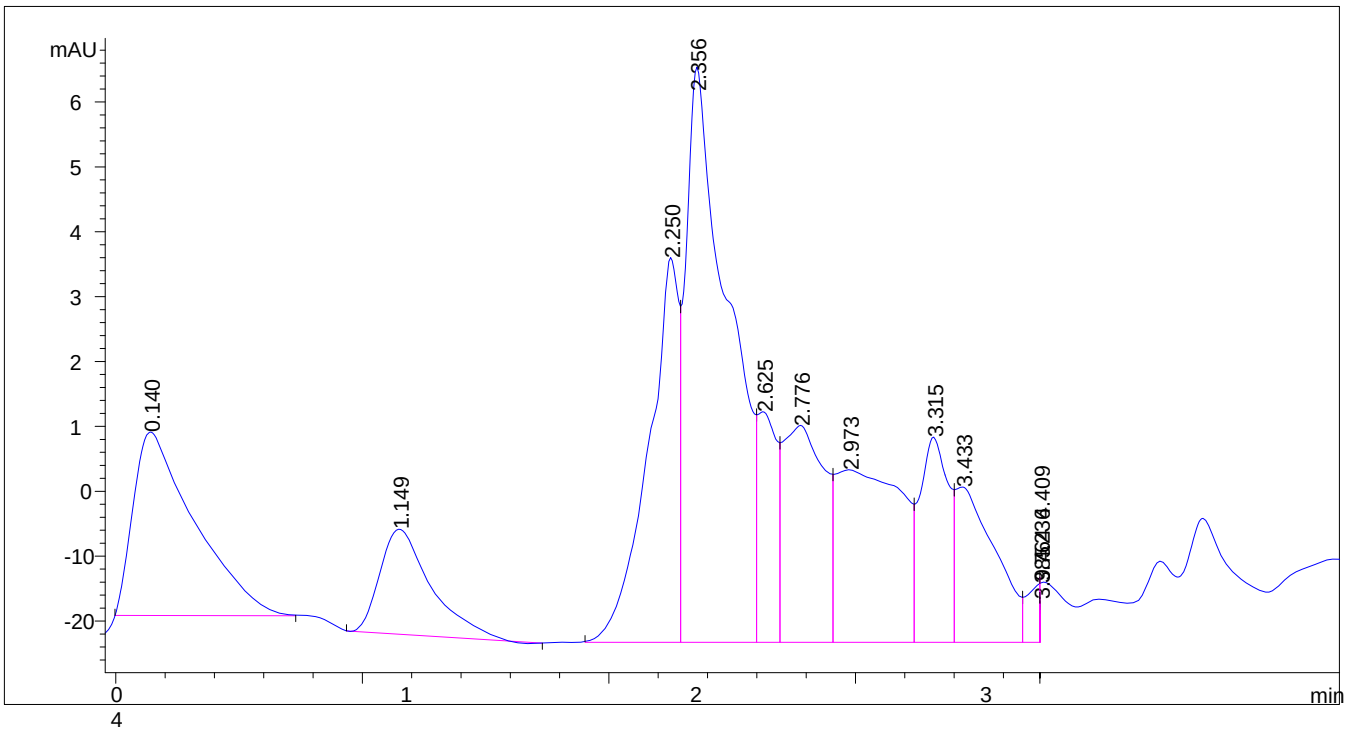
Table A.1.5 Properties of methanol

Properties	
Molecular formula	CH_4O
Molar mass	32.04 g mol^{-1}
Appearance	Colourless liquid
Density	0.7918 g/cm^3
Melting point	-97.6 °C, 176 K, -144 °F
Boiling point	64.7 °C, 338 K, 148 °F
log P	-0.69
Vapor pressure	13.02 kPa (at 20 °C)
Acidity (pK_a)	15.5
Viscosity	5.9×10^{-4} Pa.s (at 20 °C)
Dipole moment	1.69 D

APPENDIX A.2

Analysis of ascorbic acid content using HPLC

i. *C. caudatus* extracts using maceration extraction



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Area Percent

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Sorted By : Signal
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Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]
1	0.140	BB	0.2432	487.64841
2	1.149	BB	0.2132	232.07512
3	2.250	BV	0.1160	508.23740
4	2.356	VV	0.1604	1082.39612
5	2.625	VV	0.0772	192.60464
6	2.776	VV	0.1539	389.71555
7	2.973	VV	0.2395	491.43610
8	3.315	VV	0.1154	258.72708
9	3.433	VV	0.1488	268.69946
10	3.764	VV	0.1566	100.78281
11	3.985	VV	0.1623	79.21126
12	4.236	VV	0.1275	113.20190
13	4.409	VV	0.1908	270.06717
Totals :				4474.80300

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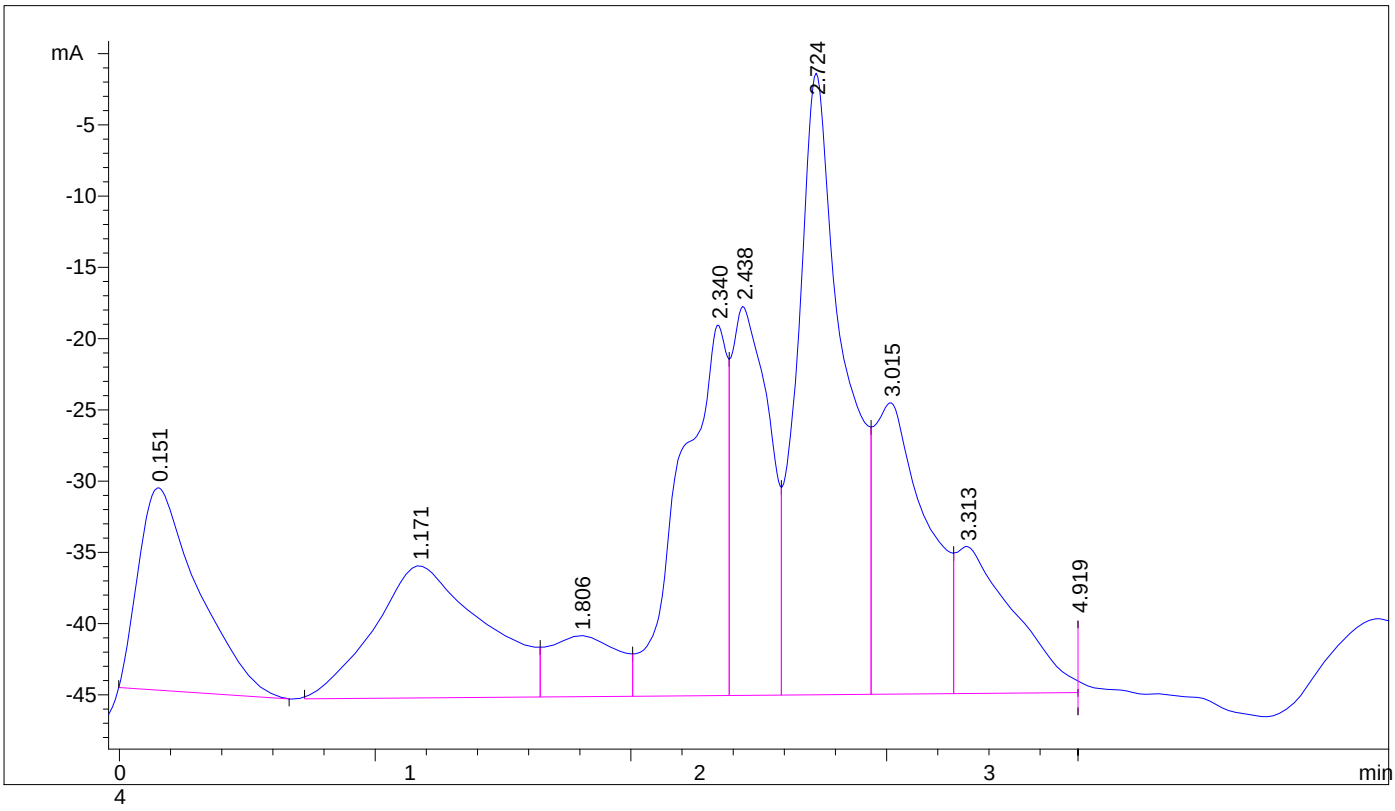
*** End of Report

ii. *C. caudatus* extracts using Soxhlet extraction

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                        Area Percent Report
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Sorted By             :      Signal
Calib. Data Modified  :      Wednesday, January 09, 2013 3:40:15 PM
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime  Type   Width     Area
  #   [min]          [min]   [mAU*s]
----|-------|------|-------|----------|
   1   0.151 BB      0.2350  229.97084
   2   1.171 BV      0.4051  279.15302
   3   1.806 VV      0.2677   80.69249
   4   2.340 VV      0.1612  323.81644
   5   2.438 VV      0.1346  277.57874
   6   2.724 VV      0.1783  569.32733
   7   3.015 VV      0.1919  294.42587
   8   3.313 VB      0.2109  167.10277
   9   4.919 BBA     0.2678  100.43391

Totals :                    2322.50140
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                        *** End of Report ***
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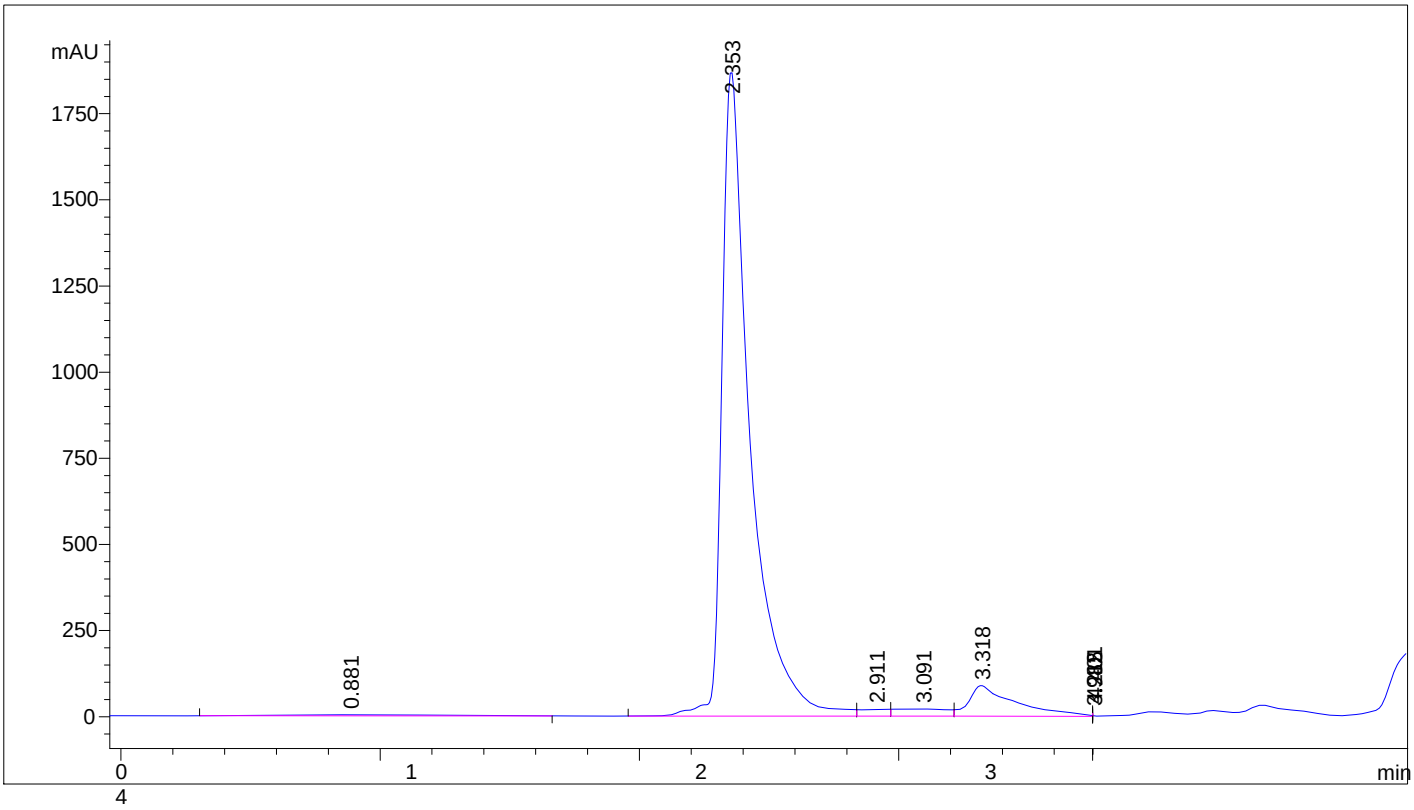
iii. *C. caudatus* extracts using ultrasonic-assisted extraction

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Sorted By             :      Signal
Calib. Data Modified  :      Wednesday, January 09, 2013 3:40:15
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime  Type   Width     Area
  #   [min]          [min]   [mAU*s]
----|-------|------|-------|----------|
   1   0.881 BB      0.5087  140.24861
   2   2.353 BV      0.1083 1712.00234
   3   2.911 VV      0.1054  153.39867
   4   3.091 VV      0.1817  291.38989
   5   3.318 VV      0.1626 1088.58081
   6   3.982 VV      0.1427  135.14484
   7   4.215 VV      0.1070  122.23196
   8   4.401 VV      0.1650  386.76651


Totals :                   4029.76363
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=                          *** End of Report
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APPENDIX A.3

Experiment Data of scavenging activity analysis

Table A.3.1 Maceration extraction data analysis

Time (hour)	Distilled water		70% acetone	
	Average absorbance	Scavenging activity (%)	Average absorbance	Scavenging activity (%)
8	0.019	95.743	0.029	93.579
16	0.029	93.723	0.039	91.631
24	0.045	90.260	0.053	88.528

Table A.3.2 Soxhlet extraction data analysis

Time (hour)	Distilled water		70% acetone	
	Average absorbance	Scavenging activity (%)	Average absorbance	Scavenging activity (%)
4	0.069	84.993	0.094	79.654
8	0.086	81.313	0.134	71.0687
12	0.072	84.416	0.105	77.201

Table A.3.3 Ultrasonic-assisted extraction data analysis

Time (minute)	Distilled water		70% acetone	
	Average absorbance	**Scavenging activity (%)**	**Average absorbance**	**Scavenging activity (%)**
30	0.135	70.852	0.171	62.915
60	0.167	63.925	0.267	42.208
90	0.120	74.026	0.228	50.722

APPENDIX A.4

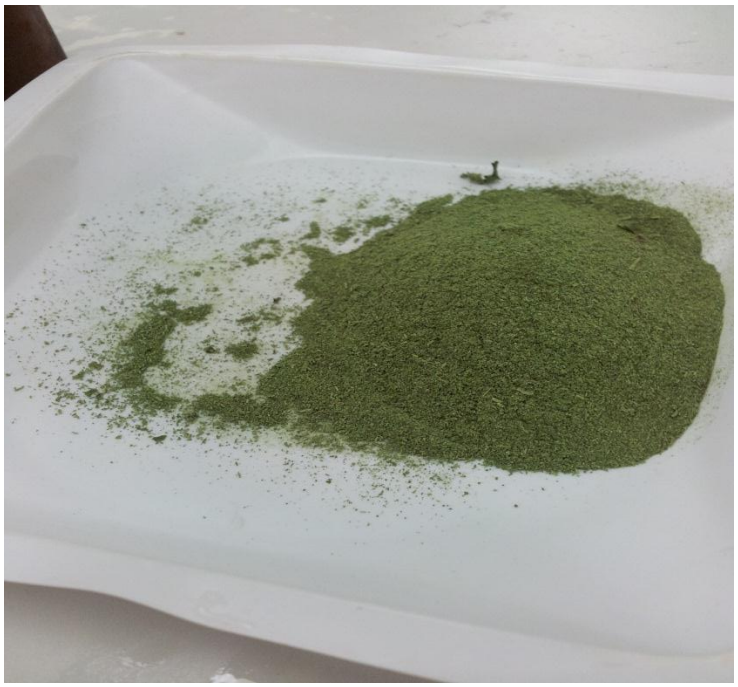

Figure A.4.1 Blended *C. caudatus*

Figure A.4.2 Filtering crude extracts

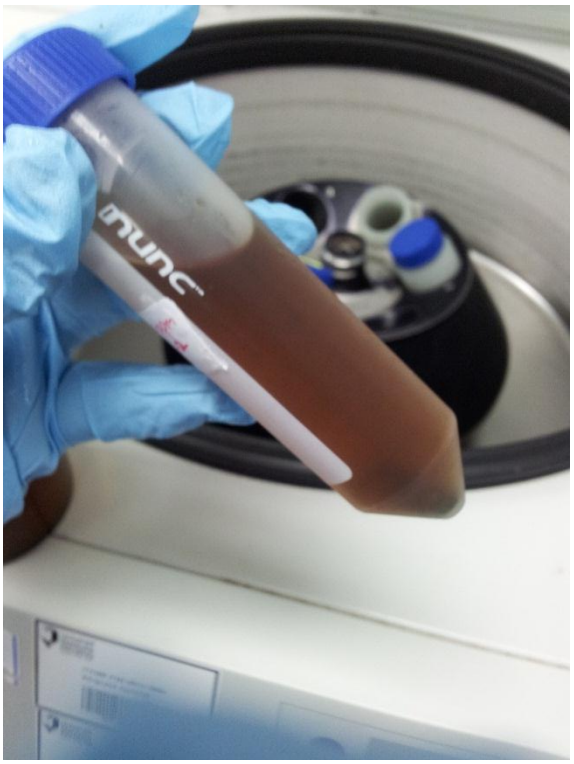

Figure A.4.3 Centrifuge crude extracts

Figure A.4.4 Separating solvent from extract