ANTIOXIDANT ACTIVITIES AND TOTAL PHENOLIC CONTENT OF *BENINCASA HISPIDA* FRUIT EXTRACTS FROM VARIOUS EXTRACTION SOLVENTS

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SUPERVISOR DECLARATION

I hereby declare that I have checked this project with title "Antioxidant activity and total phenolic content of *Benincasa hispida* fruit extract from various extraction solvents". The thesis has not been accepted for any degree and is not concurrently submitted in candidature of other degree

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STUDENT DECLARATION

I declare that this thesis with entitle "Antioxidant Activity and Total Phenolic Content of *Benincasa hispida* fruit extracts from various extractions solvents" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of other degree.

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Abstract

The effects of solvent on the extraction of antioxidants and phenolic content from *Benincasa hispida* fruit were studied by conducting the DPPH scavenging analysis and total phenol content analysis (TPC). The main idea for this study is to determine the most effective solvents that show the highest antioxidant activities and TPC from fruit. The fruit were extracted with three different solvents: methanol, ethyl acetate and hexane by using 18 to 24 hours incubation in the dark flask at ambient temperature and pressure. The efficiencies of the solvents for the extraction of the antioxidants and phenolic were in ascending order: methanol > ethyl acetate > hexane. It was found that the phenolic content of the *Benincasa hispida* in the ranged between 3 mg/l to 12 mg/l in which equivalent to the tannic acid and methanol extract shows the highest TPC, followed by ethyl acetate and hexane. The overall study found that methanol was the most effective solvent for extracting of antioxidants and phenolic.

AKTIVITI ANTIOKSIDAN DAN KANDUNGNAN FENOL DI DALAM EKSTRAK BUAH *BENINCASA HISPIDA* DARIPADA BERLAINAN JENIS PENGEKSTRAK

Abstrak

Kesan pengekstrakan terhadap antioksidan dan fenol daripada buah Kundur (*Benincasa hispida*) boleh dianalisis melalui aktiviti pengoksidaan yang terhasil dan kandungan fenol di dalam buah tersebut. Kajian ini bertujuan untuk memilih pengekstrak yang paling berkesan untuk mendapatkan aktiviti pengoksidaan dan kandungan fenol yang tinggi. Di dalam eksperiment ini, buah tersebut akan diekstrak dengan menggunakan tiga pelarut yang berbeza: metanol, etil asetat dan heksana selama 18 ke 24 jam inkubasi di dalam bekas yang gelap pada suhu dan tekanan yang normal. Kecekapan pelarut untuk mengekstrak antioksidan dan fenol adalah dalam turutan menaik: methanol > etil asetat > heksana dan didapati bahawa kandungan fenol di dalam buah kundur adalah di antara 3 mg/l hingga 12 mg/l yang dirujuk sebagai tanik asid. Tambahan pula, ekstrak daripada metanol telah menunjukkan kandungan fenol yang paling tinggi diikuti dengan etil asetat dan heksana. Kajian keseluruhan telah mendapati bahawa metanol merupakan pelarut yang paling bekesan untuk mengekstrak antioksidan dan fenol di dalam buah kundur.

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CHAPTER 1

INTRODUCTION

1.1 Research Background

An increasing health conscious in each society member, it leads the demands of healthier food product become more increased. An effective procedure is developed and used to extract the antioxidant components from *Benincasa hispida*. Most of evidence indicates that *Benincasa hispida* may protect a wide range of human condition from any disease which cause by free radical. Several mechanisms for their protective function have been proposed before, although the extraction mechanism which suite to the extraction of antioxidant is not defined yet. Basically, *Benincasa hispida* has its own antioxidant activities which influences of the metabolism production, give several effects on the synthesis and activities of intracellular enzymes and change the growth factor action. All these factors can contribute protective functionality to human health. The

mechanism will be reviewed based on their specific actions such as anti-cancer action, and cardiovascular system protective action.

Oxidants is free radical which introduced through the external and internal sources such as high exposure to the sun, pollution or radiation, stress, food intakes and etc. Actually, the molecule which loss its electron is known as free radical. This kind of molecule is not stable and will attack other molecules and attempt to share or steal their electron in order to keep it stable. This oxidation continues until key biological molecules and genes become mutated and will lowering human immune system's response to resist disease. Over time, if it is cannot be resisted, it will cause oxidative damage to wide range of tissues, organ and body system, which slowly damaging more molecule and decreasing the immune response.

Nowadays, the synthetic antioxidant is one of the sources which can be found in the supplement. Synthetic vitamins consist of synthetic chemical. Only vitamins from dietary source can be considered as nutrient for human body. Synthetic vitamins are like any other synthetic molecules but because of their antioxidant nature, they are capable to donate one electron which can break down the metabolism due to the unstable state. Hence the hydroxyl radical is produced by adding the hydrogen peroxide into human biological system. Hydroxyl radical can cause damage to DNA molecules which can leads to transform normal cell into cancer cell. Based on the situation, some action needs to be taken out to avoid the side effect of the usage of the synthetic oxidants. One of the ways is by extracting fresh food can obtain high concentration of antioxidant. There are different types of antioxidants and most of them work in paired which called synergism.

All of them work together in order to protect the body from disease by stabilizing the free radical and slowing the inevitable signs of aging.

1.2 Problem Statement

Every day, our body easily exposed to mutate and oxidized cells which cause by free radical. With a strong immune system, the human body can recognize and get rid of these cells. Basically, human body has developed a natural way to rid off of these cells through the reaction of antioxidant. In the medical field, most of researchers put a strong effort to improve the quality of food supplement from the fruits and vegetables based on traditional method which generally gives a lot of advantages to us. Based on our knowledge, no information is available on *Benincasa hispida* fruit extracts by solvent extraction method. Furthermore, for the first time, the effect of different solvents on different solvents on the radical scavenging activity and total phenolic content (TPC) of fruit extracts are evaluated.

1.3 Objectives

The aim of this study is to determine the most effective solvent extractors between methanol, ethyl acetate and hexane on antioxidant activities and total phenolic content (TPC) of *Benincasa hispida* fruit extracts.

1.4 Scopes of Project

In order to achieve the objective of this study, a few research scopes have been identified

i) Determination of extraction yield for methanol, ethyl acetate and hexane.

ii) Analyzing on the antioxidant activities of each extracts using DPPH (1,1-diphenyl-2-picryhydrazyl).

iii) Analyzing total phenol content (TPC) in *Benincasa hispida* fruit extracts

1.5 RATIONALE OF THE PROJECT

By doing this study, the effectiveness of solvents extraction on the antioxidant activities of *Benincasa hispida* can be determined. Hopefully, such study will to provide information on the antioxidant potential of *Benincasa hispida* fruit that are less well researched but nonetheless important.

CHAPTER 2

LITERATURE REVIEW

2.1 *Benincasa hispida*

Figure 2.1 *Benincasa hispida* plant

Benincasa Hispida (Thunb.) Cogn. (synonym; *Benincasa cerifera*) is one of the most valuable plants in Cucurbit family which known as Kundur (Malay), ash gourd or winter melon (English), Bhuru Kolu or Safed Kolu (Gujarati), Petha (Hindi), Kushmanda

(Sanskrit), Dôngguâ (Chinese) and Beligo (Indonesia)(Chang, *et al*.,2010). It is commonly can be found in the tropic area at below 1500 m elevations. The excessive rain may affect its yields but it still needs water especially in the early ages of development. Besides that, this herbaceous plant have thick stem which can climb up to several meters long, long leaves features and dark green fruit. Its fruit can be stuffed, steamed and cut into small pieces. It is also can be harvested about 80 to 160 days from sowing.

2.1.1 *Benincasa hispida* composition profile

Benincasa hispida is one of the thousand suggested plant species which have high potential in the modern and traditional medication to cure acute and chronic health problems. Based on Akinmoladon, *et al*.(2007) statement, there is not only mineral contain in the plants extracts and primary metabolites, but the secondary metabolites is also presence with the antioxidant properties. Generally, *Benincasa hispida* are composed of 93 percent of moisture content the weight portion, 0.3 to 0.5 percent of protein and ash of the pulp, carbohydrate, fiber and several types of vitamins (Nurul, *et al*., 2011).

Besides that, *Benincasa hispida* are suitable to plant in tropical Asia country. Table 2.1 and table 2.2 show the composition profile for *Benincasa hispida* for the

different country. Different country will contribute different composition profile of this species. It is because different countries have their own climate season such as winter, spring, autumn and summer. Malaysia essentially observes tropical weather, but the best part is it is never too hot. Humidity is a common feature, which can be duly expected from its proximity to waters. With the exception of highlands, the climate is by and large moderately hot and extremely sultry. Throughout the year, the temperature ranges from 20°C to 30°C on an average. That is why the *Benincasa hispida* which is planted in Malaysia show the highest composition in the *Benincasa hispida* compared to other country.

Table 2.1 Compositon of *Benincasa hispida* from Different Country

Country	Moisture	Protein	Carbohydrate	Fiber	Fat	Ash
Australia	98.6	0.3	1.1	1.5	0	0.3
Florida	96.2	0.4	2.24	0.68	0.003	0.45
Malaysia	94.5	0.5	4	0.5	0.2	0.3
China	96.7	0.4	2.56	0.58	0	0.27
USDA	96.1	0.4	3	0.5	0.2	0.3
FAO	96.2	0.5	2.3	0.6	0.1	0.3

(Sources: Nurul, *et al*., 2011)

Table 2.2: Vitamin Profile of *Benincasa hispida* from Different Country

Country	vitamin C	Thiamin	Riboflavin	Niacin
Australia	27	0.02	0.05	0.4
Malaysia	68	0.02	0.31	0.2
China	1.35	N.A	0.02	0.46
USDA	13	0.04	0.11	0.4
FAO	20	0.03	0.03	0.2

(Sources: Nurul, et al., 2011)

2.1.2 Contribution of *Benincasa hispida* in the Medical Experimental Development

Since *Benincasa hispida* fruit are rich with biological active compound which illustrate in the figure 2.1 and 2.2, it shows that this species can contribute in the traditional medical uses and it can be applied as anti- inflammatory, anti-obesity, anti-diabetic and many more. As seen in Table 2.3, there are two studies were developed the medical experimental from *Benincasa hispida* by using white rats.

Table 2.3: The medical experiment development

Medical Experimental	References
The fresh juice of *B.hispida* showed significant anti-inflammatory activity in cotton pellet granuloma and carrageenan induced edema in rats.	Warier, 1994
In the evaluation of anti-diabetic activity of Benincasa hispida fruit found that test compounds significantly decreases elevated level of serum glucose and also caused to reverse the cholesterol, triglyceride, HDL and LDL values when compared to untreated diabetic rats.	Raju, *et al*.,2011

2.1.3 *Benincasa hispida* World Wide Demands

With continuing demand from the customer for valuable food product which playing a vital role in health care leads the most of researchers around the world to explore and recognize the new sources of functional food ingredients. With the consumption of fruit can reduce the risk of the degenerative disease like cancer, cataract and cardiovascular diseases. The review from several scientific studies have been carried out, they reveal that *Benincasa Hispida* can be anti-inflammatory, anti-obesity, anti-diarrheal agent, anti-ulcer and antioxidant. With the high demand and the costumer awareness on the health of the Benincasa Hispida fruits, it shows that this fruit have good

economical potential. It can prove by the summarization of worldwide production of cucurbit fruits (pumpkin, squash and gourds) for the period, 1987 to 2007 byThe Food and Agriculture Organization (FAO) of the United Nations (FAO, 2009). (Nurul Aqilah, *et al*., 2011).

Table 2.4 World wide production of some important Cucurbit fruits (pumpkin, squash and gourd).

Country	Production (Metric Tons)		
	1987	1997	2007
China	1,063,366a	3,075,232a	6,309,623a
India	2,685,000a	3,300,000a	3,500,000a
Russian Federation	N.A.	750,000b	1,318,150
United States of America	N.A.	N.A.	864,180
Egypt	436,000	568,035	708,000a
Mexico	257,310	440,001	516,721
Islamic Republic of Iran	807,500b	536,000	505,000a
Cuba	N.A.	N.A.	450,000a
Ukraine	N.A.	N.A.	524,700
Philippines	N.A.	160,815	365,698
Italy	342,950	465,117	338,211
Republic of Korea	N.A.	180,779	330,040
Spain	229,921	341,309	315,000a
Turkey	380,000	381,000	337,882
Argentina	372,500	275,700	300,000a
Bangladesh	117,115	185,000	274,635
Pakistan	120,998	244,443b	255,000a
Indonesia	190,847	N.A.	254,056
Japan	276,800	247,000	237,000a
Thailand	200,000a	200,000a	226,000a

(**Sources**: FAO, 2009)

2.2 Antioxidants

Antioxidant is a molecule that can slow down or inhibit the oxidation of other molecules. Oxidation reaction happens when there is electron transfer from some substances and leads to the production of free radical. As a sequence of this reaction, these radical will start the chain reaction which can cause damage to the cell body. In order to terminate such reaction, the antioxidant play important role in such action by removing the radical and stop the oxidation reaction. Most of recent finding prove that *Benincasa hispida* also contain antioxidant agent. According to Grover, *et al*.(2001) proves that *Benincasa h*ispida extract can lowering the size of the ulcer. Besides that, Erasto *et al*.(2007) also found that *Benincasa hispida* have high potential ability to neutralize the free radical. While Beena, *et al*. (2008) found that *Benincasa hispid*a extracts can inhibit gastric mucosal injury by scavenging the indomethacin generated oxygen metabolites. Since all the finding give positive effects on the treatment, it can prove that *Benincasa hispida* can be as antioxidant agent.

2.2.1 Antioxidant Protection

Basically, oxygen is reactive atom which capable to become part of free radical. Since the free radical become main contributor for cell damaging, it can be stabilized by antioxidants before the radical attack the cell body. Hydroxyl radical, superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical and certain lipid peroxides are the examples of reactive oxygen species(ROS)(Mark, 1998).

Those ROS can be stabilized by specific antioxidant compound. Table 2 below shows the specific antioxidant compound for each ROS.

Table 2.5 Various ROS and Corresponding Neutralizing Antioxidant

Relative Oxygen Specific	Neutralizing Antioxidants
Hydroxyl radical	Vitamin C, glutathione, flavonoids, lipid acid
Superoxide radical	Vitamin C, glutathione, flavonoids, SOD
Hydrogen peroxide	Vitamin C, glutathione, beta carotene, Vitamin E, CoQ10, flavonoid,, lipoic acid.
Lipid peroxide	Beta carotene, Vitamin E, ubiquinone, flavonoids, glutathione peroxidase

(**Sources**: Mark, 1998)

Most of neutralizing antioxidant can be found in plant. Mark (1998) has already mentioned that have higher potential for inhibiting the ROS. While beta carotene with vitamin E and glutathione with vitamin C are works together to slow down the oxidation reaction. Basically, all the neutralizing antioxidant which mention above play import role to protect cell from free radical.

2.2.2 Free Radical

Molecules are consisted of atom and its electron. Basically electron present in pairs which act as bonding between each atoms. Under certain condition, that molecules have unpaired electrons due to the presence of oxidation reaction which commonly

known as free radical. Since the configuration of molecules is not stable, it will seek other electron to make it in paired via attacking other molecules.

Besides that, free radicals play an important role in a number of biological processes. Some of which are necessary for life, such as the intracellular killing the bacteria by neutrophil granulocytes. Free radicals have also been implicated in certain cell signaling processes. The two most important oxygen-centered free radicals are super oxide and hydroxyl radical. They are derived from molecular oxygen under reducing conditions. However, because of the reactivity, these same free radicals can participate in unwanted side reactions resulting in cell damage. Many forms of cancer are thought to be the result of the result of reactions between free radicals and DNA, resulting in mutations that can adversely affect the cell cycle and potentially lead to malignancy. Some of the symptoms of aging such as atherosclerosis are also attributed to free radical induced oxidation of many of the chemical making up the body. In addition, free radicals contribute to alcohol-induced liver damage, perhaps more that alcohol, itself.

2.2.3 Mechanism of Antioxidant Activities

Recent finding have already proposed that, more generative diseases are caused by free radical. Basically, the free radicals are produce continuous produce when cell respiration, metabolism and phagocytosis. Since most of us are widely expose to oxidant agent, the rate of free radical production will become increase. There are two principle of

mechanism antioxidant action which is chain breaking mechanism and ROS initiator removal (Ingold, 1968). For the first mechanism, the primary antioxidant will donate electron to the free radical. Since the first mechanism which is donating an electron to the free radical. The figure 2.1 below shows on how the polyunsaturated fatty acid (PUFA) forms a radical.

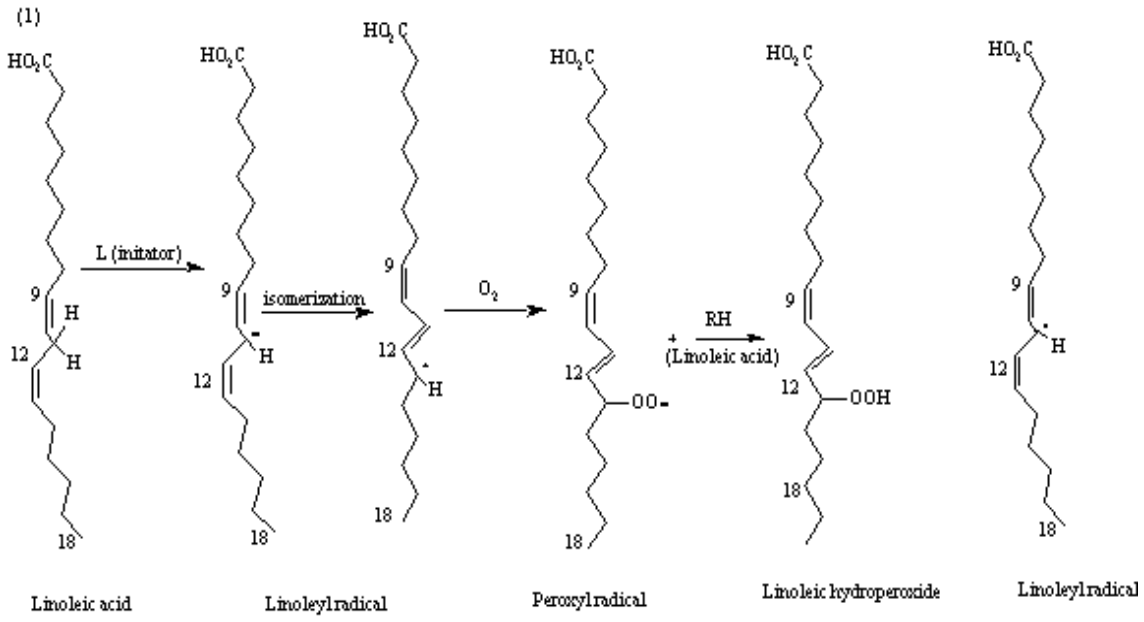

Figure 2.2 Mecahnism of linolic acid peroxidation and ROS formation

(**Sources**: Jacob and Micheal, 1999)

Since the configuration of Linoleic acid is unstable, it will undergo lipid peroxidation which can give negative sign form the human health. In order to terminate it, the first electron from the antioxidant will donate to that molecule while the second mechanism it happen when chain –initiating catalyst is quenched in order to remove the ROS initiators (Jacob and Micheal,1999).

2.3 Vitamin C as antioxidant sources

The antioxidant components are required to decrease the rate of oxidation. Most of research paper revealed that the vegetables and fruits especially *Benincasa hispida* consist of a lot vitamin c which acts an antioxidant sources. When the antioxidant level drop, it will decrease the inhibition rate and leads to cell destroys and become to be cancerous cell since the oxidative stress happened. Generally, almost 80 percent of human body covers with water and the vitamin c is one of the vitamins that soluble in water in which can travel to over part of body. In order to predict the antioxidant content in each extract, the ascorbic acid solution with the different concentrations is used as a calibration line ($r \approx 0.99$). According Sebastian, *et al*., (2003), vitamin c can donate electrons to inhibit the oxidative reaction. Once the electrons are donated, it will become a resonance-stablizef tricarbonyl ascorbate free radical (Garry and Freya, 2003). The inbition of reaction can determine by the decolourization DPPH assay. DPPH is act as a substrate to analyze the activity of antioxidants (Oyaizu, 1986). Generally, DPPH assay is in light purple solution. Once the inhibition happens, its color become fader as the vitamin c is increased.

2.4 Total Phenolic Content (TPC)

The total phenolic content is almost related to the antioxidant activity. Once the phenolic content is increased, the antioxidant activity will also increase. It also can be

found the vegetables and fruit. The tannin is one of the phenolic components in the *Benincasa hispida* fruit. Total soluble phenolics can determine by using the Folin-Ciocalteau reagent since it is soluble in the extracts and the tannic acid with the different concentration will act as internal standard in order to get approximate concentration of tannin in each extracts.

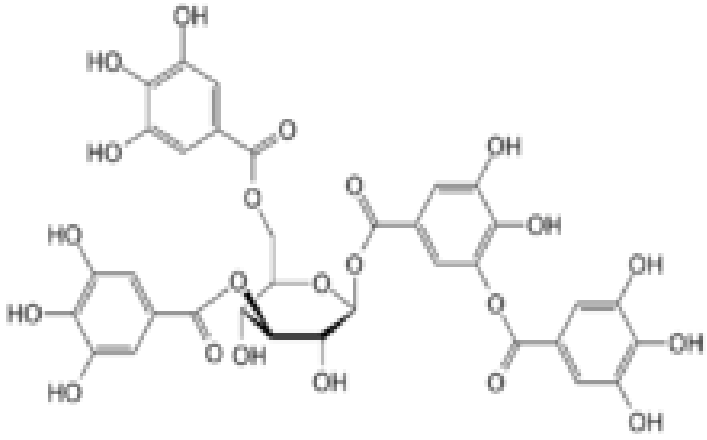

Figure 2.3 Chemical Structure of Tannin

2.5 Sample Preparation for Medical Plant analysis

2.5.1 Sample Preparation

Sample preparation is the crucial procedure in the medical plant analysis since there has been increasing interest worldwide in the used of medical plant as other alternative for preventing and treating of various illnesses. The extraction and

characterization of active compounds from medicinal plants have resulted in the discovery of new drugs with high therapeutic value (Donehower and Rowinsky, 1993). Thus, the sample preparation technique is developed which gives more advantages compare to the conventional method such as distillation method. The distillation method is the method of separating mixtures based on differences in volatility of components in a boiling liquid mixture. Generally, this process is involved of separating mixture with high range of temperature; it will decrease effective or destroy the bioactive compound in the plant. There are several modern techniques that have been developed such as solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction and microwave- assisted extraction for preparation of medical analysis (Carmen, 2002).

2.5.2 Extraction method

2.5.2.1 Traditional Extraction method

Solvent extraction is one of the traditional extraction methods which used organic solvent as the reactant. For the plant extraction, solvent extraction method is frequently used for isolation of antioxidant compound (Bushra, *et al*., 2009). Various solvent can be used for the extraction but the polar organic solvent is the most prefer as extractor. The success of this method depends on antioxidant compound characteristic and polarity of each solvent (Bushra, *et al*., 2009). Liquid-liquid extraction is based on the transfer of a solute from one liquid phase into another liquid phase. It is become useful tool if the

researcher chooses suitable solvent extract method which can separate the selective substance from mixture or remove unwanted impurities.

2.5.2.2 Solvent used

In this experiment, there are three solvents used in order to get *Benincasa hispida* extracts. The solvents are commonly chosen in the extraction process. Table 2 below shows the comparison between the properties of this solvent (Knovel Critical Tables (2nd Edition), 2008).

Table 2.6 Solvent Properties

Solvent	Formula	Boiling point (°C)	miscibility	Polar Index
Methanol	CH_3OH	64.7	Miscible in water and organic solvent	5.1 (polar)
Hexane	C_6H_{14}	69	Immicible in water but miscibile in organic solvent	0.1(non polar)
Ethyl acetate	$CH_3COOCH_2CH_3$	76.5	Miscible in water and organic solvent	4.4 (polar)

(**Sources**: Knovel Critical Table (2nd Edition), 2008.)

2.5.2.2 Modern Extraction Method

There are four suggested modern extraction method for preparing the medical plant analysis which are supercritical-fluid extraction (SFE), microwave-assisting extraction (MAE), pressurized liquid extraction (PLE) and pressurized hot water extraction (PHWE). The description of these methods is mention as follow:

(a) Supercritical- fluid extraction (SFE) method

The purpose of SFE method is to extract the biological compound using supercritical dioxide instead of chemical solvent. It can be used either for the sample preparation step or on a larger scale production. The sample is placed in an extraction vessel and pressurized with CO_2 to dissolve the sample. The contents are depressurized and the CO_2 loses its solvating power causing the desired material to precipitate. The condensed CO_2 can be recycled (retrieved from: http://www.waters.com/waters/nav.htm).

There are several advantages for the SFE method which are the extraction of active compound are more efficient and selective without thermo degradation, it is suitable for extracting the compound with low concentration in which have high volatility and induced less racemization than solid-liquid extraction(Carmen, 2002).

(b) Microwave-assisting extraction (MAE) method

MAE method is a method of heating solvents in contact with a sample which involved microwave energy to partition bioactive compound from the sample matrix into the solvent. In this method, the closed vessels which content the mixture of sample and solvent are directly exposed to the heat which cause by microwave energy at any temperature (Brian, 1994). The purpose for this method is to reduced the time taken for extracting the sample and can be conducted in large amount of sample (Carmen, 2002).

(c) Pressurized-liquid extraction (PLE) method

PLE method is method for extracting the bioactive compound from mixture of sample and solvent. Basically, it is works similar as Soxlet extraction, but this method operates at the high pressure and temperature that above point of the solvent. The uses of higher temperature increase the ability to solubilize the bioactive compound, decrease the viscosity of solvents, allowing the better penetration of the solvent into sample matrix while the uses of higher pressure will facilitate the extraction of bioactive compound from the sample by improving the solvent accessibility to the bioactive compound that is trapped in the matrix pores (Valeria, *et al*., 2006). By applying this method, it can reduce the extraction time and extract the higher amount of bioactive compound.

(d) Pressurized- hot water extraction (PHWE) method

PHWE method is one of the method options for extracting bioactive compound. The purpose for this method is to reduce the uses of solvent which instead by the utilization of pressurized water at elevated temperature and controlled pressure condition (Chin *et al.*, 2010). Since this method is only used water as its extracting medium, the cost of extraction will be reduced and become efficient for less polar organic components.

Besides that, Chin, *et al.(*2010), states that this method is required pressurized hot water which is denoted as the region of condensed phase of water between 100°C to 374°c. . During the extraction, moderate pressures are needed to keep a condensed phase of water such as 15 bar at 200◦C and 85 bar at 300◦C. If the pressure decreases below the boiling point at any pressure, superheated steam will be formed.

2.5.3 Challenges in Analysis of Plant Active Component

Basically, the active component is located between of the matrix structure of the mixture. It is required to perform specific method for isolating that active component form the matrix. There are several considerations which should take seriously before conducting the extraction of active component such as temperature, pressure, time taken for extraction and the solvent used. While performing the extracting, it was required to

undergo at the optimum temperature and pressure in order to make sure that the target bioactive component are not destroy. Besides that, the selection of solvent extract is one of the main problems for the extraction. Generally, the selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products.

2.6 Effect of solvent on the extracting of antioxidant and phenolic compound

The main key of solvent extraction was depended on the polarity. The polarity of each solvent used in such study had been showed in the Table 2.6. The higher the polarity value, the more tendencies extracts were produced. Hence, the results obtained among the solvents were found that, the methanol had higher tendency to extract more components followed by ethyl acetate. Generally, the methanol had hydroxyl group which can form intermolecular hydrogen bonding with the antioxidant and phenolic components which can increase the solubility rate (Silla, *et al*., 2011). The ethyl acetate was considered in the carboxyl group due the presence of carbonyl bond which had less polar compare to the methanol. Hexane was the poorest solvent extraction due to the presence of covalent bonding. Table 2.7 below shown the supporting evidence for the result was obtained before with the various species of plants.

Table 2.7 Analysis of Result of Various Solvent Extractions from Difference Species of Plant

No	Plant Species	Analysis Solvents Extraction result	Reference
1	*Chromolaena odorata*	For the vitro inhibition was scored in methanol extracts of *C. odorata* which offered highest inhibition zone against tested bacteria E. *coli*, *S. aureus*, *X. vesicatoria* and *R. solanaccearum*, respectively followed by ethyl-acetate and hexane extracts.	Sukanya, *et al*.(2011),
2	*Symplocacea e sp.*	Successive extractive values revealed that methanol (8.8%) obtained higher percent of yield followed by ethyl acetate (5.7), hexane (2%) and chloroform (2%). in the leaf powder.	Lakshmi and Vadiru (2010),
	Dillenia Indica L.	(59.99±2.21 mg/g) was recorded in 50 % aqueous methanolic extract which also shows highest antioxidant activity with IC50 value of 56.66±1.55 µg/ml.	Baikuntha, *et al*. (2012),
4	*Marrubium peregrinum*	Methanolic exstract of *M. peregrinum* showed the highest phenolic and flavonoid concentration and strong antioxidant activity.	Milan (2010)

CHAPTER 3

MATERIALS AND METHODS

3.1 Chapter Overview

This chapter discussed the materials and methods adapted in the experimental work. A schematic structure of the whole process flow has been conducted and illustrated in Figure 3.1.

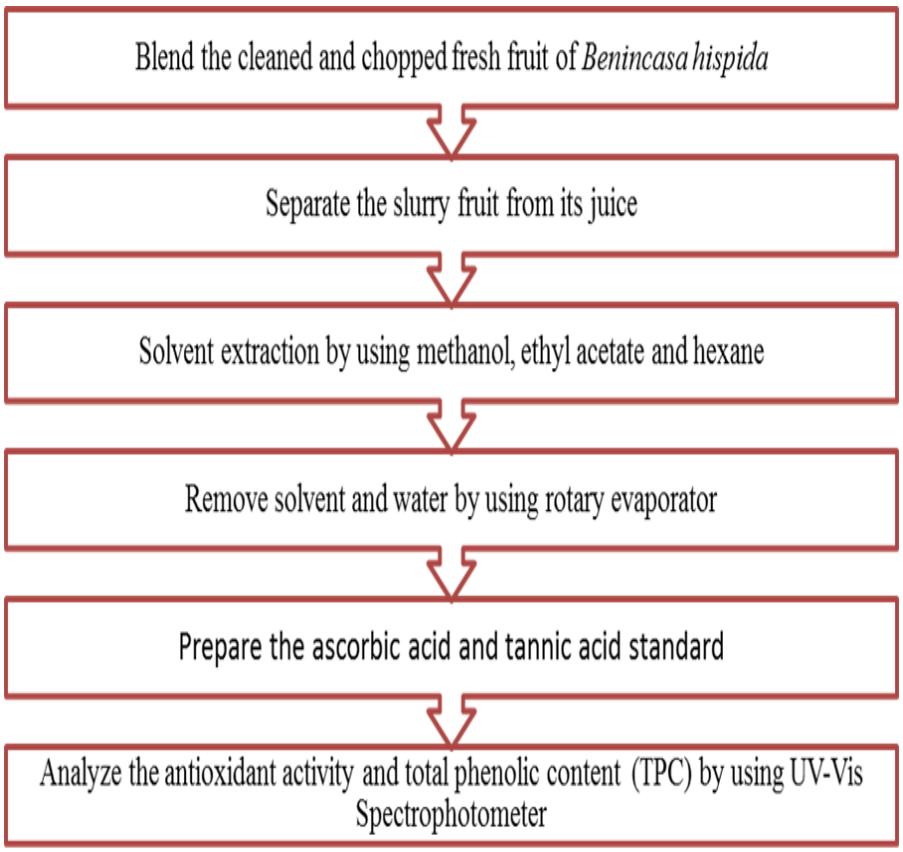

Figure 3.1 Process flow of overall methodology

3.2 Materials

3.2.1 Chemicals

The solvents used in this experiment: methanol ethyl acetate and hexane were in the HPLC grade. 1, 1-diphenyl -2-picrylhydrazyl (DPPH), Folin and Ciocalteau reagent, sodium carbonate (Na_2CO_3), butylated hydroxyl toluene (BHT), standard of vitamin c (ascorbic acid) and standard of phenolic acid (tannic acid) were listed in the analysis chemical used. All listed chemical were purchased from Sigma Aldrich.

3.2.2 Plant Material

The mature *Benincasa hispida* fruit were collected fresh from Warisan Kundur Resources in Pekan, Pahang. 200 grams of fresh fruit were required for experimental purpose.

3.3 Methodology

3.3.1 Preparation of samples extract

the *Benincasa hispida* fruit were obtained, peeled and chopped into small size. The chopped fruits were then blended in a food processor to produce uniform slurry and followed by filtering the slurry to separate it from the juice. 200 g of slurry fruits were prepared in three 500 ml of dark conical flask as the preparation for extraction process.

In the extraction process, the ratio of the fruits and solvent extractor is 1: 1 which about 200 g of slurry sample and 200 ml of solvent. Then, 200 ml of each solvent was transferred into the conical flask that contained 200 g of slurry fruits and followed by 18 to 24 hours incubation at the temperature. After 24 hours, the infusions were filtered through Whatman No.1 filter paper with the assisting by vacuum filter apparatus and the residue was re-extracted with equal volume of the same solvent used before. After 48 hours, the same steps were repeated. Once the extraction was done, the obtained supernatant was kept in the 4°C. Such extraction procedure mentioned above was modified from Total Phenolic Content, Flavoid Concentration and Antioxidant Activity of *Marrubium peregrinum* L. Extracts journal (Millan, 2011). The extraction of Benincasa hispida fruit were required to be done for four times for obtaining precise data.

In order to concentrate the extract, it was required to remove almost the solvent which were methanol (boiling point: 64.7°C), ethyl acetate (boiling point: 76.5 °C) and hexane (boiling point: 69°C) and small amount of water present by using rotary evaporator. Basically, rotary evaporator consisted of a heated rotary flask in which maintained under a vacuum through a tube with connecting it to a condenser. The rotating flask which contained the extract was heated by partial emersion in a hot oil medium. The undesired solvent and water were evaporated and the leave the flask by the connecting tube and condensed in the condensation section. Then, the condensate was flowed into other flask which was connecting after the condensation tube. Here, the speed of rotary evaporator and temperature was set at 3 rpm and 100°C.

Figure 3.2 Rotary evaporator

3.3.2 Determination of DPPH (1,1-diphenyl -2-picrylhydrazyl) free radical Scavenging

This procedure was modified for measuring antioxidant in each extracts based on DPPH free radical scavenging procedure developed by Brand-Williams *et al.*, 1995.

Firstly, the stock solution was prepared by transferring 25 mg of DPPH into a dark flask which contained with 100 ml methanol. The solution was stored at 4 °C for preserving it before conducting the analysis.

Then, the stocks need to dilute about 1:10 on methanol. 0.1 of each of extract samples solutions were added into 3.9 ml diluted DPPH solution to complete 4 ml reaction medium. In this experiment, BHT was act as controller for identifying the antioxidant in each sample. The reaction was completed about 20 minutes in dark and ambient temperature. The absorbance is measured at 515 nm in a UV-Vis. All modified steps were repeated for four times for obtaining precise data.

In this study, the scavenging activity was represented by the inhibition percentage data. The inhibition data of each extract sample was calculated from the decrease of absorbance according to the formula which referref from Margitanová et al., 2012:

$$\text{Inhibition (\%)} = \frac{(Ao - A_1o)}{Ao} \times 100 \qquad (3.1)$$

Where;

Ao = DPPH working solution absorbance

A_1o= DPPH absorbance after adding the extracts

3.3.2.1 Ascorbic Acid Standard Curve

The ascorbic acid standard curve was prepared: 1000mg/l of stock solution by dissolving 10mg/ml ascorbic acid in 10 ml of methanol. Then, the stock solution was diluted to 10, 20, 30, 40, 50, 60, 60, 70, 80, 90 and 100 mg/l (Sonkar and Mishra, 2011). The absorbance value for each concentration was obtained by using UV-Vis with 515 as their wavelength. The dilution of this solution was done for four times. Here, the calibration curve (n=4; r=0.981) was obtained. Those absorbance values can estimate the extract concentration. . The concentration of antioxidant for each sample was expressed in mg of Ascorbic acid equivalents (AAE) / g of extract**.**

3.4 Total Phenolic Content (TPC)

Total phenolic content of the extracts was measured using the Folin-Ciocalteau spectrophotometric method which was reported by Singleton et al (1999) with some modification. This method is based on measuring color changes that caused by reduction

of Folin-Ciocalteau reagent by phenolates in the presence of sodium carbonate (Na_2CO_3).

In this method, 1 ml of each extracts was added to 5 ml of 0.2 N Folin-Ciocalteau reagents. Then, it was required to stand about 5 minutes for proper reaction between extract and reagents. 4 ml of 37.7 g/L of sodium carbonate ($Na_2CO_{3)}$ were added and the total volume made up to 25 ml by using distilled water. The above solution was kept for incubation at room temperature for 2 hours. Absorbance was measured at 760 nm using 1 cm cuvette in a perkin-elmer UV-VIS lambda 25 spectrophotometer. The determination of TPC was carried out in triplicate and expressed as means ± standard deviation.

Besides that, tannic acid (0 - 800 mg/L) was used to obtain standard curve for the phenolic content. The absorbance value for each concentration was obtained by using UV-Vis with 760 nm as their wavelength. The dilution of this solution was done for four times. Here, the calibration curve (n=4; r=0.997) was obtained. Those absorbance values can estimate the phenolic in sample.The total phenolic content was expressed in mg of Tannic acid equivalents (TAE) / g of extract.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

This chapter discussed the experimental results that carried out in the research work. The material discussed in this chapter includes the effect of solvent on antioxidant activity and phenolic content of *Benincasa hispida* fruit by determined the yield of extraction, DPPH radical scavenging activity and total phenolic content. Then, each of the results are discussed thoroughly and justified accordingly.

In order to determine the most effective solvent extractor between methanol, ethyl acetate and hexane for this experiment, there were two analysis methods which were DPPH analysis and total phenolic content (TPC) analysis that had been conducted to each *Benincasa hispida* fruit extracts. In DPPH method, 1-diphenyl-2-picrylhydrazyl was used as reagent which can indicate the rate of scavenging acidity for each sample.

The purpose for this analysis is to determine the antioxidant activity by comparing the rate of inhibition for each sample with BHT which acts as controller. While the determination of total phenolic content in each extracts was the second analysis in order to full fill the requirement of the objective for this study. For the TPC analysis, the changing of Follin-Ciocalteau reagent color which caused by phenolates in the presence of sodium carbonate were the indicator to analysis the phenolic in the sample.

Once the extraction was done for about 48 hours, it was found that there were two differences between each extracts which were the volume of mixing extracts with each solvent and the presence of oil layer which know as fatty acid. Those differences can be referred at the figures 4.1(a), figure 4.1(b) and figure 4.1(c). Based on those figure, it was shown that methanol had the higher volume of mixture which about 245 ml of mixture and followed by 230 ml of ethyl acetate mixture and 210 ml of hexane mixture. For the second observation showed that, the presence of fatty acid layer. Hexane had form a thickness layer of fatty acid compare to ethyl acetate. It was because hexane can remove polar lipid (Walter and Purcell, 1979). While, in the methanol mixture, there was no layer had formed after extraction. The thicker layer of fatty acid form, the less volume mixture was produce.

Figure 4.1(a): Methanol extracts

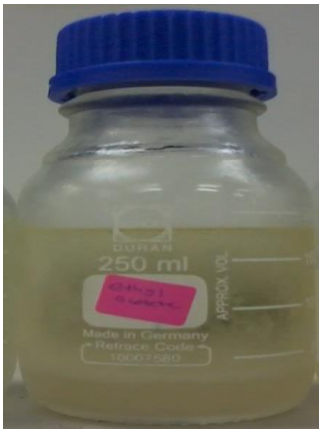

Figure 4.1(b): Ethyl Acetate extracts

Figure 4.1(c): Hexane extracts

4.2 Yield of prepared extracts

The effect of different polarities of solvent on the yield of extraction formed was calculated by the following formula:

$$\text{Yield of extraction (\%)} = \frac{\text{weight of dried extract (g)}}{\text{Weight of original sample (g)}} \times 100\ \% \qquad (4.1)$$

Extracts of fresh *Benincasa hispida* fruit were obtained following a sequential solvent extraction procedure and yield for each solvent was calculated separately (Table 4.1). Noordin et. al.(2012), found that hexane gave the highest yield of extracted solvent from *Hibiscus cannabinus L.* seed. Similar results were observed in the present investigation. The highest yield was obtained for hexane (47.24 %) followed by ethyl acetate (37.78 %) and methanol (23.12%) respectively. Such a wide variation in the yield of extracted is due to the differences polarities of the extraction solvent.

The data obtained for extraction yield was expressed as means ± standard deviation and showned in Table 4.1.

Table 4.1 Yield of extracted fresh *Benincasa hispida* fruit (m*(mean ± standard deviation))

sample extract	Yield of extract (%)
Methanol	23.12 ± 0.654
ethyl acetate	37.78 ± 1.312
Hexane	47.24 ± 0.476

The final weight of each sample was obtained after the residue dried about 24 hours at 60°C. Difference polarities of solvent gave different weight of sample. In this study, figure 4.2 was shown the final weight of sample extracted. The range of dried sample was obtained in the range of 40 to 100 g. The weight of dried extracted sample were represented in ascending order; methanol (46.25 ± 1.309 g) followed by ethyl acetate (75.56 ± 2.625 g)and hexane (94.49 ± 0.953 g) since methanol had the highest polarity value compared to ethyl acetate and hexane. The higher the polarity value, the more tendencies extracts were produced. Hence, the results obtained among the solvents were found that, the methanol had higher tendency to extract more components followed by ethyl acetate. Generally, the methanol had hydroxyl group which can form intermolecular hydrogen bonding with the antioxidant and phenolic components which can increase the solubility rate (Silla, *et al*., 2011). The ethyl acetate was considered in the carboxyl group due the presence of carbonyl bond which had less polar compare to the methanol. Hexane was the poorest solvent extraction due to the presence of covalent bonding.

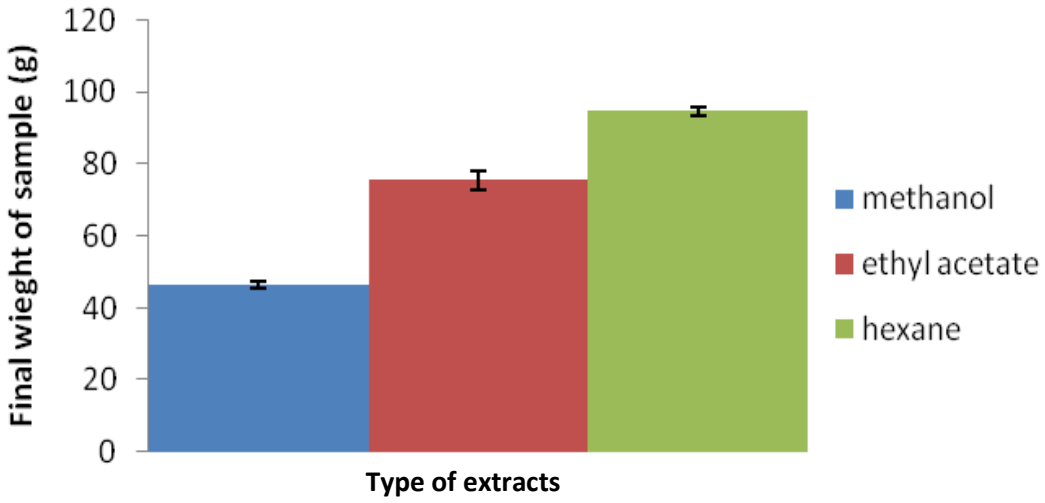

Figure 4.2: The final weight of sample obtained after extraction

4.3 Antioxidant Activity analysis

Generally, DPPH assays were very sensitive to the light source and easy to perform test. Since, such assays had stable free radicals, it can decolorize in the presence of antioxidant. It was visible in the light purple which can measure its absorbance in 515 nm wavelength. DPPH assays had provided the reactivity of the compounds with stable free radicals due to the presence of odd number of electrons (Raju, *et al*.,2005). The absorbance of the DPPH assays after adding the samples were measured for 20 minutes. Once this assays receive the electron from present antioxidant, the color of the assays were turned into pale yellow. This model of scavenging the stable DPPH radical was a

commonly used method to evaluate the free radical scavenging ability of various samples (Lee, *et al*., 2003). In this study, the effect of scavenging activity was represented by the inhibition rate and calculated based on the following formula:

$$\text{Inhibiton (\%)} = \frac{(Ao - A_1o)}{Ao} \times 100 \qquad (4.2)$$

Where;

Ao = DPPH working solution absorbance

A_1o= DPPH absorbance after adding the extracts

In this method, 0.541 ± 0.003367 was obtained for DPPH working solution absorbance, Ao. This value was act as a reference absorbance in order to determine the scavenging activity for each solvent.

The percentage of inhibition rate has a relationship with the concentration of antioxidant which expressed as ascorbic acid equivalents (AAE) in each samples. All the data was shown in the Table 4.2 and showed that, when the antioxidant of sample present was higher, the percentage of inhibition rate was also higher. Antioxidants can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress (Majumdar, 2012). The present antioxidant of each sample was shown in ascending order: methanol > ethyl acetate > hexane > BHT (standard).

Table 4.2: The relationship between concentrations of antioxidant obtained with the inhibition rate for each sample table. (m*(mean ± standard deviation))

Sample	Concentration of Antioxidant /AAE (mg/l)	Inhibition Rate (%)
Methanol	22.56 ± 0.33	38.72 ± 0.77
Ethyl Acetate	9.31 ± 0.47	5.68 ± 0.60
Hexane	3.78 ± 0.47	3.55 ± 0.57
BHT	0.90± 0.27	1.89 ±0.095

According to Margitanová et al.(2012), when the antioxidant was increased it will fade the DPPH color from light purple into colorless. Once the DPPH solution was faded, it was effect the absorbance value for each sample and the concentration of antioxidant can be obtained from ascorbic acid standard line (the standard line equation; $y= 0.08x + 0.159$, $r^2 = 0.981$). For this analysis, it was carried out for four trials. The absorbance value for methanol, ethyl acetate, hexane and BHT were obtained about 0.3395 ± 0.0026, 0.2335 ± 0.003, 0.18925 ± 0.004 and 0.16625 ± 0.0022. While the concentration of antioxidant data was obtained for each extracts were based on the absorbance value obtained and followed by the percentage of inhibition rate. When the absorbance value of sample was higher, the concentration of antioxidant was also higher and leads the percentage of inhibition became more increased. Thus, based on the statement above, it was found the highest of antioxidant activity is methanol extracts. In this experiment, a control with DPPH assay without extracts was performed. Synthetic antioxidant; butylated hydroxytoluene (BHT) were used as positive control. The level of antioxidant activity was observed same as other extracts. When compared with the synthetic antioxidant, BHT, all extracts offered higher antioxidant activity. The order of antioxidant activity was shown as follows: Methanol> ethyl acetate > hexane > BHT. To

the best of our knowledge, no earlier report is available regarding the antioxidant activity of fresh *Benincasa hispida* fruit with which to compare the result of our present analysis.

4.4 Total Phenolic Content (TPC) Analysis

The phenolic compounds in plant extract were often associated with other molecules such as proteins, polysaccharides, terpenes, chlorophyll and inorganic compounds (Anil, *et al*., 2012). For the reference component of total phenolic content in *Benincasa hispida* was tannin. Tannins are natural polyphenols can classified into condensed tannins (proanthocyanidins) and hydrolyzable tannins (gallo- and ellagitannins) (Yizhong, *et al*., 2004).

Total phenolic content of the extracts was measured using the Folin-Ciocalteau spectrophotometric method which was reported by Singleton, *et al*.(1999) with some modification. This method is based on measuring color changes that caused by reduction of Folin-Ciocalteau reagent by phenolates in the presence of sodium carbonate (Na_2CO_3).

In this study, the TPC data was expressed in terms of tannic acid equivalent (the standard line equation; y= 0.161x, r^2 = 0.997.. The TPC of the *Benincasa hispida* fruit value varied over a wide range between 3 mg/l to 12 mg TAE/l, among the extracts prepared in different solvent (Figure 4.3). It was found that the highest concentration of phenols was measured in methanol (11.32 ± 0.20 mg/l), followed by ethyl acetate (8 ±

0.33 mg/l) and hexane (3.3 ± 0.18 mg/l) extracts. The finding reveals that the most of TPC in fresh *Benincasa hispida* are highly polar in nature, and thus more efficiently extractable by polar solvents. Moreover, finding is in agreement with the observation of Mattaus (2002), who found that the high efficiency of polar solvents (water and methanol) for extracting TPC from several oilseeds.

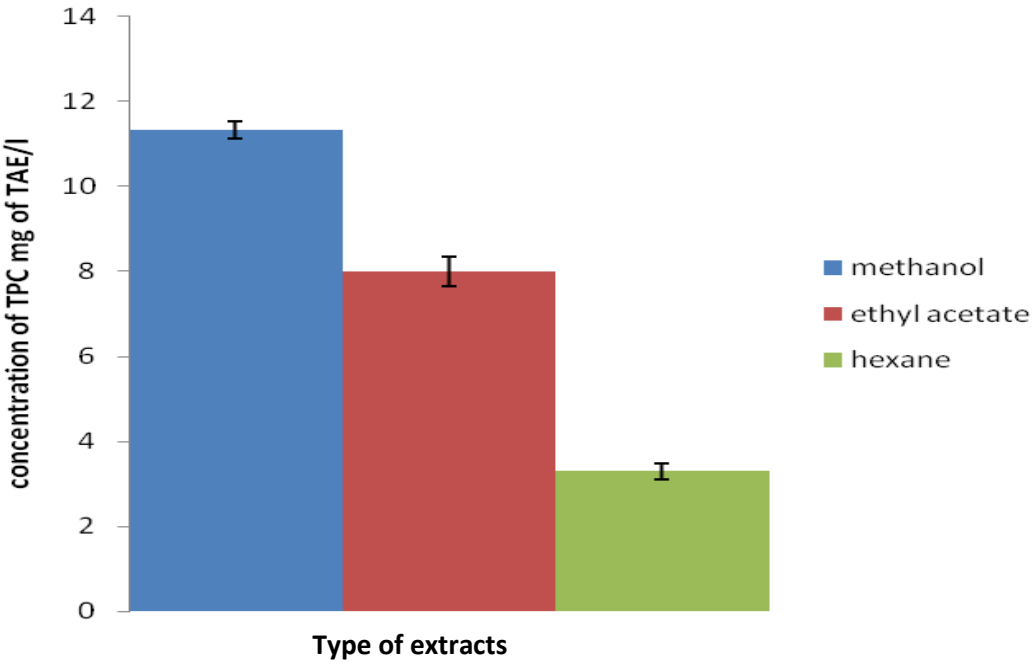

Figure 4.3 The concentration of phenolic / TAE (mg/l) obtained.

4.5 Relationship between Antioxidant and Total Phenolic Content (TPC)

The phenolic compounds in the *Benincasa hispida* extracts had the relationship with the antioxidant properties. The relationship of antioxidant and total phenolic content data were found based on 4.3 and 4.4 analysis. Based on both analyses, it was found that, the total phenolic data was increased as the antioxidant increased.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Experimental study has been carried out to achieve the objective of the study. The objective for this study is to determine the most effective solvent for extracting antioxidant and total phenolic content (TPC) between methanol, ethyl acetate and hexane.

In this study, *Benincasa hispida* fruit was chosen as plant which rich of antioxidant and phenolic compound. It is because, there is lack of data obtained which related to the fruit compared to the other part of that species.

In order to achieve the objective above, there are three analyses that had been conducted to each of the extracts sample which are the solvent efficiencies for extracting samples, the antioxidant activity and TPC.

From the results of the present study, it can be concluded that the methanol is the most efficient solvent to extract the antioxidant and phenolic compound in the *Benincasa hispida* fruit. It is because, methanol can perform the highest efficiencies of solvent extractor, antioxidant activities and TPC for four trial of the experiment..

5.2 Recommendation

In order to conduct further study that related to antioxidant activity of *Benincasa hispida*, there are two recommendations were being proposed:

1) Perform in vivo studies to make further confirmation if the observation in the vitro activities that have been done can be replicated in vivo

2) Since the *Benincasa hispida* fruit is lack of profile data, there are several parameter should be study such as effect of pH, temperature and concentration in order to optimize the antioxidant activity.

3) In order to analyzing the effect of polarity of the solvent, the sample should be test with the different ratio of solvent.

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APPENDIX A STANDARD DATA

concentration (µg/ml)	v1 (stock solution)ml	methanol (ml)	absorbance (515)				Averange trial	Standard deviation
			trial 1	trial 2	trial 3	trial 4		
0	0	10	0.137	0.149	0.154	0.148	0.147	0.007164728
10	0.1	9.9	0.207	0.214	0.194	0.215	0.2075	0.009678154
20	0.2	9.8	0.31	0.298	0.314	0.302	0.306	0.007302967
30	0.3	9.7	0.424	0.416	0.434	0.425	0.42475	0.00736546
40	0.4	9.6	0.541	0.542	0.539	0.545	0.54175	0.0025
50	0.5	9.5	0.654	0.662	0.659	0.645	0.655	0.007438638
60	0.6	9.4	0.764	0.761	0.756	0.743	0.756	0.009273618
70	0.7	9.3	0.811	0.816	0.813	0.799	0.80975	0.007455423
80	0.8	9.2	0.883	0.885	0.873	0.89	0.88275	0.007135592
90	0.9	9.1	0.915	0.909	0.913	0.915	0.913	0.002828427
100	1	9	0.989	0.992	0.993	0.999	0.99325	0.004193249

Table A.1 Ascorbic Acid Standard Data

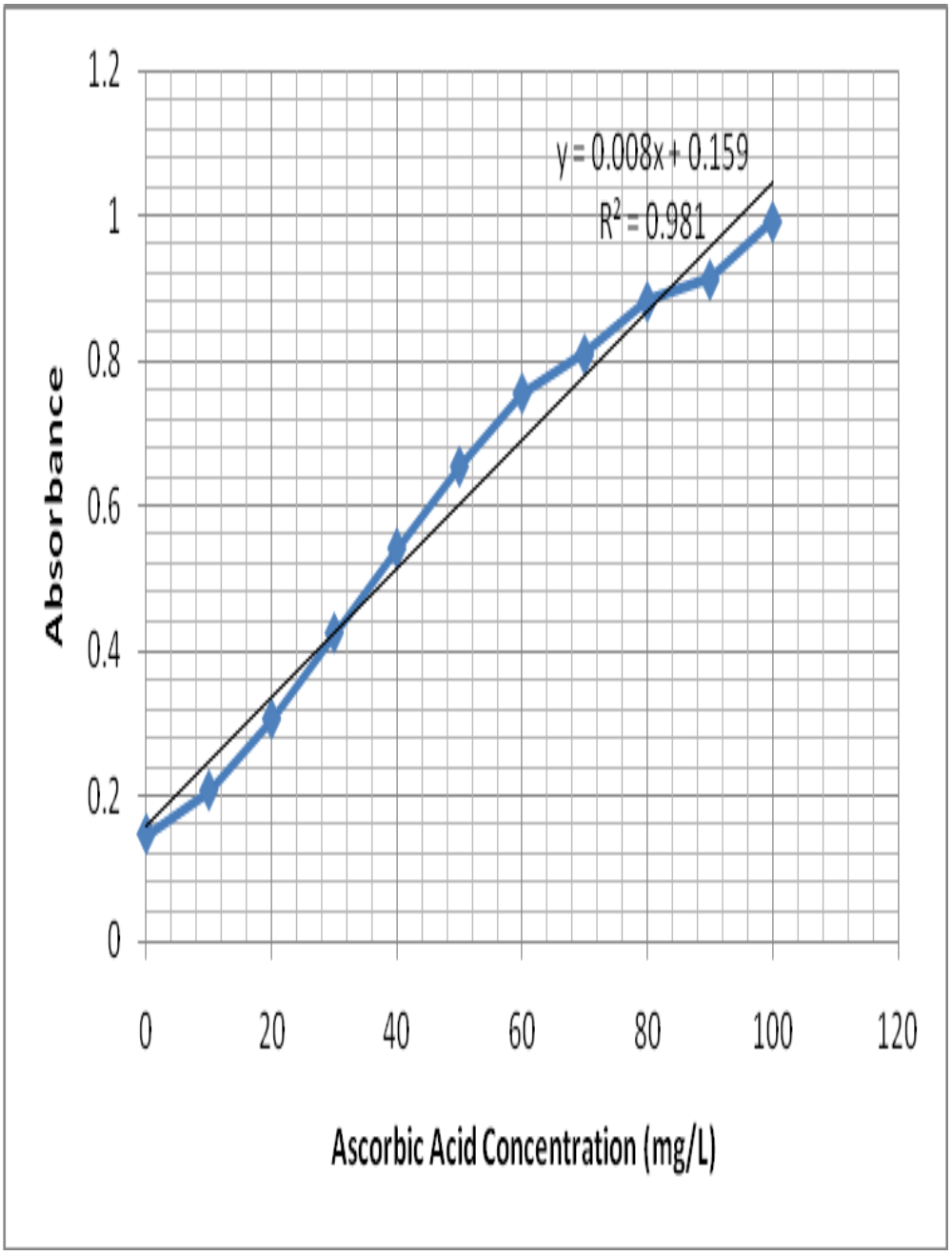

Figure A.1 Ascorbic Acid Standard

	Absorbance (OD)						
Concentration (mg/mL)	trial 1	trial 2	trial 3	trial 4	Average	STDEV	STDEV (%)
0	0	0	0	0	0	0	0
0.2	0.034	0.031	0.029	0.03	0.031	0.00216	0.21602469
0.4	0.07	0.07	0.071	0.071	0.0705	0.000577	0.05773503
0.6	0.091	0.089	0.094	0.09	0.091	0.00216	0.21602469
0.8	0.132	0.132	0.129	0.13	0.13075	0.0015	0.15
1	0.16	0.159	0.162	0.161	0.1605	0.001291	0.12909944
1.2	0.181	0.181	0.182	0.179	0.18075	0.001258	0.12583057
1.4	0.231	0.229	0.23	0.231	0.23025	0.000957	0.09574271
1.6	0.258	0.257	0.26	0.259	0.2585	0.001291	0.12909944
1.8	0.293	0.292	0.291	0.292	0.292	0.000816	0.08164966
2	0.325	0.323	0.322	0.326	0.324	0.001826	0.18257419

Table A.2 Tannic acid Standard Data

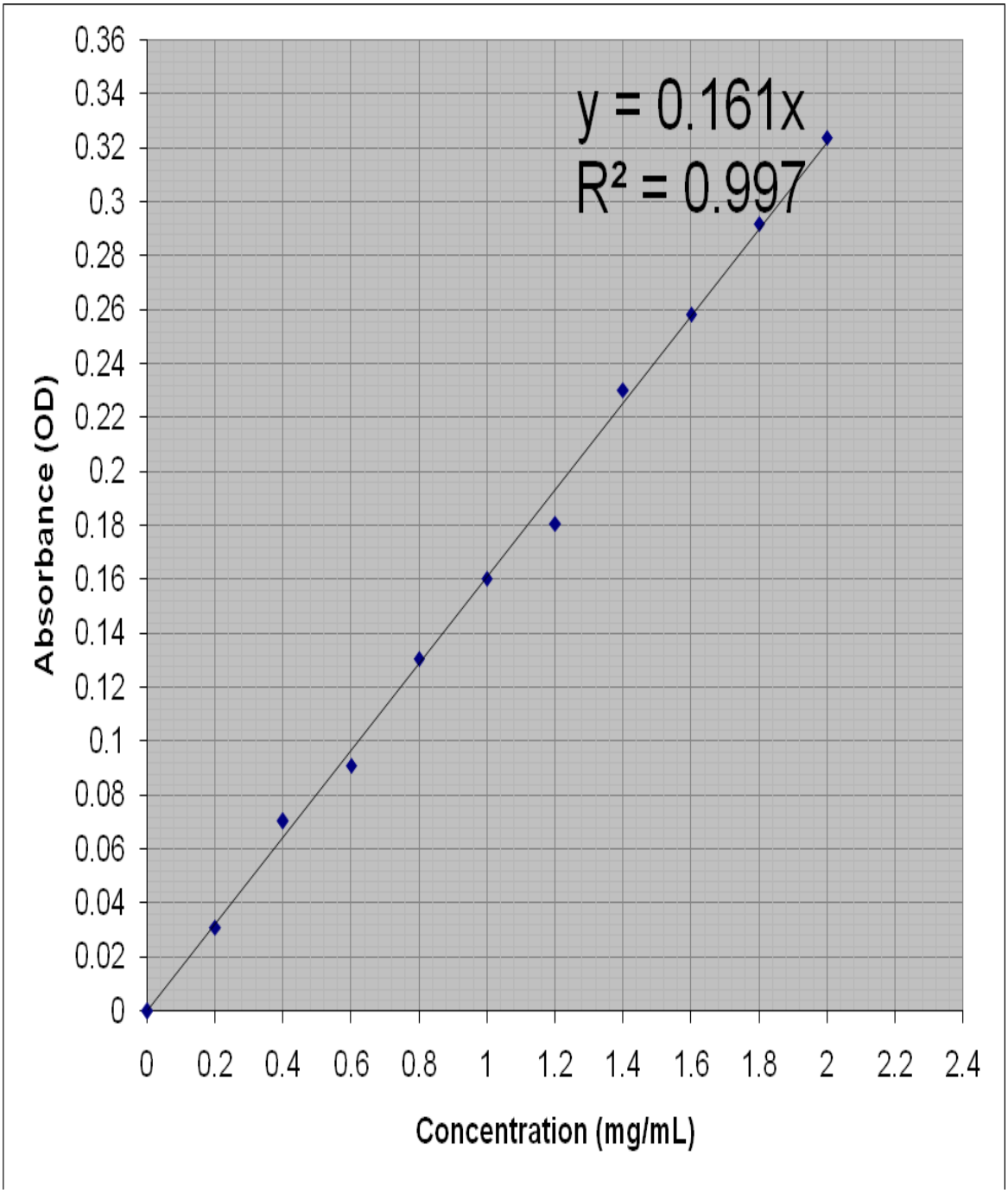

Figure A.2 Tannic Acid Standard