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

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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.

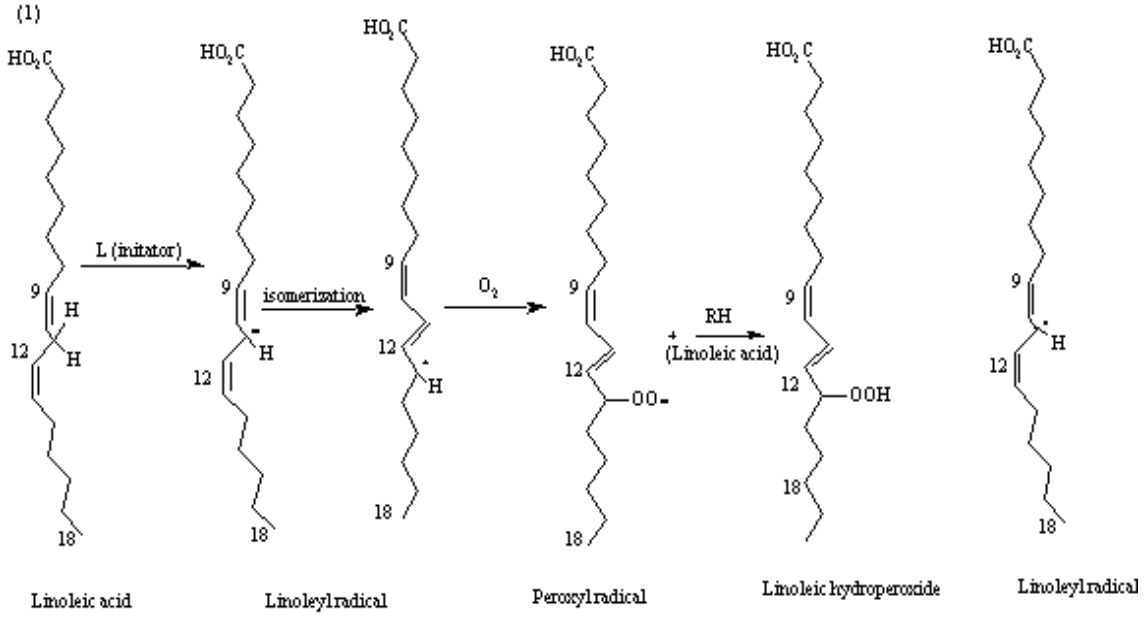

Figure 2.2 Mecahnism of linolic acid peroxidation and ROS formation

(**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.

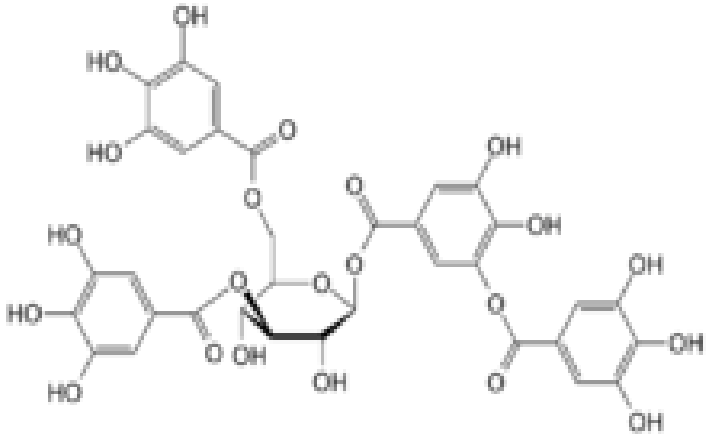

Figure 2.3 Chemical Structure of Tannin

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