ANTIBACTERIAL AND ANTIHYPERTENSIVE PROPERTIES OF CRUDE EXTRACTS OF IN VITRO CALLUS CULTURE OF MUSKMELON

(Cucumis melo L.)

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ABSTRACT

Muskmelon or *Cucumis melo* L. is one of the melon families that are widely consumed in many countries. The plant’s ability to adapt to many climate conditions makes it available in the market throughout the year. Phytochemicals are one of the important components in plants. In this study, the methanolic extract of callus from muskmelon were studied and tested for antihypertensive and antibacterial activities. The explants were chosen from plant parts; leaf, stem and fruit according to the highest total phenolic content where the leaf part showed the highest content. The calli were induced from the explant in the medium with plant growth regulators (PGRs) at a concentration ranging from 0 to 2.0 mg/L. Murashige and Skoog (MS) medium supplemented with PGRs: Zeatin (Z) 1.5 mg/L with 2,4-Dichlorophenoxyacetic acid (2,4-D) 1.5 mg/L, 6-Benzylaminopurine (BAP) 0.5 mg/L with 2,4D 1.5 mg/L, Indole-3-Butyric acid (IBA) alone 1.5 mg/L and 2,4-D alone 1.5 mg/L have successfully induced viable callus for extraction. The four weeks old calli were chosen for methanolic extraction because of its high total phenolic (TP) and total flavonoid (TF) content. It was found that C4 contained the highest amount of TP and C3 showed highest TF. The methanolic extracts were tested for antibacterial and antihypertensive activity. Three Gram Negative bacteria *Escherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC 1331), *Pseudomonas aeruginosa* (ATCC 1542), and three Gram Positive bacteria *Enterococcus faecalis* (ATCC 14506), *Staphylococcus aureus* (ATCC baa1026), and *Bacillus subtilis* (ATCC 11774), were used for antibacterial test in this study. Only the C3 and C4 extracts were able to inhibit bacterial growth as measured by inhibition zone and minimal inhibitory concentration. While in antihypertensive test, the C4 extract showed the highest inhibition rate (70.32%) against angiotensin converting enzyme and the extract from C3 showed the lowest (50.78%) inhibition. The phytochemical profile of the extracts was determined by HPLC where the presence of phenolics and flavonoids were detected in some of the extracts.
ABSTRAK

Muskmelon atau *Cucumis melo* L. adalah salah satu tumbuhan dari famili Melon yang di makan dengan meluas di kebanyakan negara. Kebolehannya untuk menyesuaikan diri dengan banyak keadaan iklim, menyebabkan tumbuhan tersebut boleh berada di pasaran sepanjang tahun. Fitokimia adalah salah satu komponen terpenting dalam tumbuhan. Dalam kajian ini, fitokimia dari ekstrak kalus Muskmelon dikenalpasti dan ekstraknya dikaji untuk aktiviti anti-hypertensi dan anti-bakteria. Explant diambil dari beberapa bahagian, daun, batang dan buah untuk memilih bahagian tumbuhan yang mempunyai kandungan jumlah phenolik yang tertinggi, dimana daun menunjukkan kandungan jumlah phenolik yang paling tinggi. Jadi, kalus dihasilkan daripada daun di dalam medium yang mempunyai pengatur pertumbuhan (PGRs) di dalam kepekatan 0 hingga 2.0 mg/L. Medium dengan penggalak tumbuhan (PGRs): Zeatin (Z) 1.5 mg/L dengan 2,4-Dichlorophenoxyacetic acid (2,4D) 1.5 mg/L, Benzylaminopurine (BAP) 0.5mg/L dengan 2,4D 1.5 mg/L, Indole-3-butyric acid (IBA) 1.5 mg/L dan 2,4D 1.5 mg/L yang telah berjaya mengaruhkan pertumbuhan kalus yang sesuai untuk diekstrak. Kalus yang berumur empat minggu dipilih untuk diekstrak dengan pelarut kerana kebanyakannya mempunyai kandungan fenoliks (TP) dan flavonoid (TF) yang tinggi. Ekstrak kalus C4 mempunyai kandungan TP paling tinggi dan ekstrak C3 mengandung kandungan TF paling tinggi. Ekstrak metanol kalus tersebut telah dikaji untuk aktiviti antibakteria dan antihipertensi.Tiga Gram-negatif: *Escherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC 1331) *Pseudomonas aeruginosa* (ATCC 1542), dan tiga Gram-positif bakteria: *Staphylococcus aureus* (ATCC baa1026), *Bacillus subtilis* (ATCC 11774), dan *Enterococcus faecalis* (ATCC 14506) telah digunakan dalam eksperimen ini. Dalam kajian antibakteria, hanya ekstrak kalus C3 dan ekstrak kalus C4 dapat membunuh bakteria berdasarkan diameter zon penyekatan dan kepekatan minima yang dapat membunuh bakteria. Kemudian, dalam kajian antihipertensi pula, ekstrak kalus C4 menunjukkan kadar penyekatan 70.32% terhadap enzim angiotensin. Ekstrak kalus kultur dari C3 menunjukkan kadar penyekatan paling rendah sebanyak 50.78%. Fitokimia ekstrak dikaji oleh HPLC yang mana fenolik dan flavonoid telah ditemui dalam kandungan beberapa ekstrak.
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min 	Minute
MS 	Murashige and Skoog
N 	Nitrogen
NaOH 	Sodium hydroxide
Kg 	Kilogram
PGR 	Plant growth regulators
pH 	Hydrogen Ion Concentration
r 	Error
rpm 	Revolutions per minute
s 	Second
SH 	Schenk and Hilderbrandt medium
TLC 	Thin layer chromatography
UV 	Ultraviolet
UV-B 	Ultraviolet –B
W 1 	First week
W 2 	Second week
W 3 	Third week
W 4 	Fourth week
v/v 	Volume per volume
V 	Volume
Z 	Zeatin
2,4D 	2,4-Dichlorophenoxyacetic acid
μl 	Microlitre
μg 	microgram
% 
Percentage
°C  Celsius Degree
CHAPTER 1

INTRODUCTION

1.1 STUDY BACKGROUND

Nowadays, studies of polyphenols are becoming increasingly important in order to find any potential medicinal activities from natural resources. There are synthetic drugs produced in the market in order to lessen bacterial infection or cancer diseases, but most of them contain many side effects to human. Thus, it is important to find the natural source to treat those problems from our daily diet sources.

Researchers are eager to discover the medicinal potential of the edible plant and organisms. The environment is rich with ample supply of both biological and chemical diversity. This diversity provides a unique combination of compounds with the demand potential in the industry such as cosmetic, pharmaceuticals, and nutritional supplements where each of these products has potential market values.
1.2 MUSKMELON (*Cucumis melo* L.)

Muskmelon (*Cucumis melo* L.) is from Cucurbitaceae family, which might have potential antibacterial and anticancer activities. Cucurbitaceae family includes cucumber, squash, watermelon and cantaloupe. There are a diverse group of melon that include orange flesh cantaloupe, mixed flesh honeydew and mixed melon (Henry *et al.*, 2012). It provides aroma, color and variety of Malaysian diet. Melon fruits are high in vitamin C, beta carotene (Adam and Richardson, 1981), and it is widely consumed in the countries.

A study previously done by Vouldoukis *et al.*, (2004), found that the muskmelon pulp extract contained anti-inflammatory and high antioxidant properties. The high antioxidant activity was found in the leaf and stem extracts of muskmelon (Hajar *et al.*, 2009). Antioxidant plays a role in the human body to fight against free radicals, or ageing which can help to treat cell’s injuries. Callus culture is used as the subject in this study because it can provide unorganized cell masses, rapid and uniform access to nutrition and precursors. Thus, callus culture can provide alternative sources to study antibacterial and antihypertensive activities.

Plant cell culture is a useful tool to study plant molecular biochemistry and molecular biology in basic stage. Plant cultures can provide a simplified model system for the study of plants when compared with the original plants or differentiated plant tissue culture in the study of cellular and molecular processes. In biochemical and molecular investigation of plant secondary metabolite, cell culture can also be used as the subject of choice (Dicosmo and Misawa, 1995).

The production of useful compounds by plant cell cultures has become increasingly important, as an example, the production of polyphenols which have potential values to be developed and enhanced for antibacterial and antihypertensive activities. Often, cell suspension cultures consist of a homogeneous cell population, which concede accelerated and uniform access to nutrition, precursors, growth hormones and signal compounds of the cells (Mustafa *et al.*, 2011).
The study of plant compound had been done previously for its potential activities against cancer cell or microbial. The production of secondary metabolites has become increasingly important for medicinal interest. However, the study for plant secondary metabolite such as phenolics content or flavonoids may need ample supply of plants under controlled conditions, free from microbes and insects, plant that yields specific metabolites, organic substances that are extractable and low cost of maintenance.

Plant cells, cultured in vitro have been considered to have a potential source of specific secondary metabolites. In order to extract secondary metabolite of plant, cell cultures can be utilized for obtaining large scale of plant source production of secondary metabolites (Mercedes et al., 2010). It also can provide a continuous, reliable source of plant sample that can be determined and use for bioactivity studies.

### 1.3 OBJECTIVES

1) To optimize the concentrations of plant growth regulators (PGR) for callus induction of Muskmelon (*Cucumis melo* L.)

2) To determine the total phenolics and total flavonoids content and chemical profile of methanolic extract of Muskmelon (*Cucumis melo* L.) callus.

3) To determine the antibacterial and antihypertensive activity of methanolic extract of Muskmelon (*Cucumis melo* L) callus.
CHAPTER 2

LITERATURE REVIEW

2.1 IMPORTANCE OF MEDICINAL PLANT

Plants benefiting men in many ways, including preventing from diseases, maintaining health or cure ailments (Fasihuddin and Ghazally, 2003). Human and animal depends on the plants for their food and sources of pharmaceutical drugs in medicine. Plants also known to produce metabolites for their defense against microorganism, insect and herbivores. It was reported that 80 % of the world population had depended on medicinal plant for their primary health care (Vines, 2004).

It was reported in many ancient civilizations, such as Egypt or Chinese had provided strong evidence regarding use of medicinal plant to cure many ailments (Hubner and Munstedt, 2009). The plant contains many important secondary metabolites that can be utilized as medicine. But, due to overexploitation of the plant source in order to search for medicinal plant, the plant may face extinction. Aloe vera L. (Liliaceae) had long been used to treat cuts as it is applied to skin itches, cuts and burns. The sap is also used to relieve stomachache and as a hair shampoo (Fasihuddin and Ghazally, 2003). Muskmelon had also been used for curing ailments as alternative medicine such as to cool down the body heat, lower the blood pressure and eczema.
Figure 2.1: Graph showing the publications of medicinal plants and traditional medicine from 1965 - 2005 (Adapted from Janick and Whipkey, 2007).

Figure 2.1 shows number of publications from 1965 - 2005 on medicinal plants. It shows an increasing trend in numbers of studies done on search of medicinal plants from years to years. In 2005 to 2006, there was a rise in price of plant extracts from medicinal plants in the international market (Sindhu, 2004). Plant tissue culture can help to produce mass production for sources of plant extracts, this can lower the cost for producing the extracts.
2.2 CUCURBITACEAE: THE GOURD FAMILY

It is reported that there are 825 species and 118-119 genera from the Cucurbitaceae family (Jeffrey, 2005). The plant included cucumber, watermelon, muskmelons, squash, and pumpkins. For most of these plants, the fruits are often gigantic and very delicious. It is medium sized plants, primarily found in warmer regions of the world. Cucurbitaceae is one of the major families of economical importance, particularly the plant species with edible fruit such as muskmelon, watermelon, cucumber, and pumpkin (Gebhardt et al., 1982; Kocyan et al., 2007). The family is morphologically and biochemically differ from other families and therefore considered monophyletic.

There are many plant species under Cucurbitaceae family. Most of these plants are herbaceous, climbed plant and perennial (Lassnig, 1997). The plant species also have an extensive root system which ramifies the root system. The stems are glabrous, usually hairy or prickly and the vascular bundles are bicollateral. Often the leaves are large, deeply lobed spirally arrange on long petioles and simple. The cucurbitacins is the compound that usually presents in Cucurbitaceae family, which sometimes gave the bitter taste (Okoli, 1984). The plant species are rarely affected by environmental changes, so the anatomical structures of the plants are useful for taxonomic characterization of the plant (Stace, 1980). The plants are also highly conservative in taxa variations.

In cultivation of cucurbits, they are usually very sensitive to frost. So, in the cold climate, the crop may need to begin growing indoors with shady place to avoid frost and intense sunlight with the help of cocopeat before they can be grown in soil outdoor. After 10 days of germination from seed, they can be grown on the grown to reduce root damage and high concentration of phosphorus must be applied to the plant to increased nutrient levels after transplanting. This is because, after transplant, they cannot maintain their nutrient levels, unless they are grown in situ. The cucurbit plant can grow better in in warm condition with rich nutrient soil and moist at the same time. They need long days of light...
and a lot of moisture in with good plant condition. Usually they will produce edible fruit after planting by 48-56 day (US Department of Agriculture, 1977).

There are many nutrition and uses of cucurbits. Family cucurbits are considered as one of the most healthy foods. As an example, in cucumber, it is composed of 96 % of water with little fiber and few calories which makes it as the prime food to loose weight. It also provide sources of Vitamins with large amount of potassium that has been identified to have certain properties of cancer preventive benefits (American Cancer Society, 2006).

The other benefits are they provide beneficial nutrients and minerals. Thye contain high levels of Vitamin B, iron, magnesium and phosphorus. They also have an even high levels of Vitamin C, E, potassium, copper and manganese. As an example, the pumpkin flesh has a good source of dietary fiber which is good for digestion system. In daily value for average diet, Cucurbits can provide 148 % of Vitamin A in 100 gram.

2.3 MUSKMELO
Scientific Classification: *Cucumis melo* L.

Kingdom: Plantae
Order: Cucurbitales
Family: Cucurbitaceae
Genus: *Cucumis*
Species: *C. melo*
Binomial name: *Cucumis melo*

(Renner and Schaefer, 2008)

Scientifically, *Cucumis melo* L. was classified as Cucurbitaceae family, which includes bitter melon, pumpkins and cucumber. Many of the plants of Cucurbitaceae family can be found to provide food that are usually been consumed. It was indicated that the plant is highly nutritious for human health (Ouzounidou *et al.*, 2006).
2.3.1 Origin and distribution

Figure 2.2 shows the Muskmelon plants. Muskmelon is also widely known as rock melon, cantaloupe, Persian melon, netted melon and melon (USDA, 2008). There are various varieties of muskmelon and according to FAMA (2008), the ‘‘Glamour’’ and ‘‘Goodies’’ variety is the most popular in Malaysia. The varieties can be differentiated based on individual fruit color, shape, size, fruit pulp and fruit sculpture.

It is nearly the same as honeydew, but what makes it different from other melon is its skin, fruit’s color and its musky aroma. It is reported that muskmelon comes from Central Africa, but some said that it originates from Persia (MARDI, 2008).

![Muskmelon plants](source: www.gmpeladang.com.my)

**Figure 2.2: Cucumis melo** L. plants. (Source: www.gmpeladang.com.my)

Muskmelon was introduced into Malaysia since year 2000. It increasingly becomes popular and there is high demand for the fruits in the market, although it
fetches high prices and low crop production in certain areas. It encourages the entrepreneurs to grow the muskmelon plant.

It is a plant of high commercial value and had been cultivated in many countries because of its abilities to adapt to variable climate conditions. Now, it is widely grown in the tropics, subtropics and the temperate regions. The best condition is to grow the plant with well aerated and sandy soil in weed free condition (Zulkarami et al., 2010). In Malaysia, they are commercially planted as one of the economic fruit crops in the east coast. Each plant of muskmelon can produce 4-5 fruits (Chan and Lok, 2005). Figure 2.3. shows the Muskmelon fruit. It is conventionally grown using seeds, which are expensive and have high risk of fungal infection. In vitro production of the plant is seen as one of the solutions to lower the production cost and still the technology need to observe further (Villanueva et al., 2004).

![Muskmelon Fruits](Source:www.fitho.in)

**Figure 2.3:** *Cucumis melo* L. fruits (Source:www.fitho.in)
2.3.2 Nutritional and medicinal value of muskmelon

According to the previous findings by Ouzounidou et al., (2006), there are high content of organic and mineral nutrients in the melon. However, it is also depending on its cultivar and cultivation method. Fertigation method is highly recommended in the crop production of melon because it can produce high quality and high yield of fruits.

The levels of the macronutrient of melon were also affected by the cultivation method and cultivar according to the observation carried out in 2004-2007. The important nutrients present in the muskmelon are phosphorus, calcium, potassium, magnesium, iron and copper, vitamin A and C, folate, pyridoxine (vitamin B₆), nicotinamide (vitamin B₃), and dietary fiber (Gene, 1997). Vitamin A and C are both powerful antioxidants, they help to protect the body tissues against oxidative damage by free radicals.

Active and passive smokers are advisable to consume a piece of the fruit daily because vitamin A is known to protect the lungs and prevent inflammation. It can offer some protection against lung cancer. Muskmelon is a good source of beta-carotene, which is a precursor for vitamin A.

Eating muskmelon can help to improve vision. If it is often consumed, it helps to reduce the chances of developing early cataract. Its high fiber content can help to prevent constipation. It is also low calorie value and one cup of muskmelon contains about 71 calories (Gene, 1997). It also reduces the absorption of fat in the gastrointestinal tract and control cholesterol levels in blood. It is believed that eating muskmelon, can lead to lower cardiovascular problems. The table 2.1 shows phytochemical screening of methanolic extract of muskmelon seed. It is found that it contains high alkaloids and triterpenoids. There are minor contents of flavonoid, carbohydrates, protein and phytosterol. Table 2.2 shows some plant compounds exist in some plants with its bioactivity.
Table 2.1: Phytochemical composition of *Cucumis melo* (Methanolic seed extract)

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterol</td>
<td>+</td>
</tr>
</tbody>
</table>

++ = high degree of presence, + = low degree of presence

(Source: Arora *et al.*, 2011)

2.4 PLANT TISSUE CULTURE

2.4.1 *In vitro* culture

*In vitro* culture is one of a technique of plant tissue culture to propagate plant which can produce many plantlets from a single explant. It is very similar with plant propagation through an asexual cycle (Gaba *et al.*, 2004). The plants produced are usually perpetuated because during normal cell division (mitotic), genes are typically copied exactly at each mitotic division (Malepszy, 2004).

Plant tissue culture is widely used in the plant cultivation industry as well as in the plant production organization. The method is well known after the discovery of plant growth regulators. It has been reported that some melons have been propagated by *in vitro* culture techniques (Kim *et al.*, 1997). Chan and Lok (2005) had successfully regenerated *in vitro* plantlets from nodal segments of melon (honey dew). In this study, the *Cucumis melo* callus extracts had been used to test for bioactivities where it never been done by previous study.

There are basically five stages of *in vitro* propagation of plantlets. There are stages 0, i, ii, iii, iv. Stage 0 involved plant selection and stage I preparation. Stage II,
the production of suitable propagule where the production of new plantlets is capable of giving rise to intact plants. Stage III is prepared for growth in the natural environment and stage IV is transferring the plant to the natural environment.

There are advantages and disadvantages involved with plant tissue culture. The main advantage is the culture can be started using small pieces of explants. So, in order to maintain the plants, only a small space is required. In plant tissue culture, the environment can be controlled or altered to meet specific needs of the plant to be grown and plantlet can be produced all year round (Tsay and Mulabangal, 2003). This is because the plant was grown in *in vitro* environment.

The genetically improved plant can be produced by alternating the genetic of the plant with desired characteristic and it can be produced in large scale by tissue culture. Plant tissue culture is also useful for conservation of threatened plant species as it will only need a small part of the plant to regenerate whole plant.

The plant produced is usually free from diseases, bacteria, fungi and other microorganism as the propagation is carried out in aseptic condition. There are various methods available for producing virus free plant which is important in the crop industry (Mohiuddin *et al.*, 1997).

One of the disadvantages is that the plant tissue culture required advanced skills in order to produce well grown propagated plants. Moreover, the expensive and specialized facility is needed to obtain optimum results from plant species and variety which needed specific methods to be applied. When introducing the young plantlet to the external environment, it will be more susceptible to water loss. This is because in internal environment, plantlet is grown with a high level of relative humidity. The plantlets need to be hardened in external environment with slowly decreased humidity.