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ANTIBACTERIAL AND ANTIHYPERTENSIVE PROPERTIES OF CRUDE EXTRACT OF *IN VITRO* CALLUS CULTURE OF MUSKMELON (*Cucumis melo* L.)

NURUL ASHIKIN BINTI ABDUL KARIM

MASTER OF SCIENCE (BIOTECHNOLOGY)

UNIVERSITI MALAYSIA PAHANG

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NURUL ASHIKIN BINTI ABDUL KARIM

Thesis submitted in fulfilment of the requirements
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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Master of Science in Biotechnology.

Signature

Name of Supervisor: Dr. Natanamurugaraj Govindan

Position: Senior Lecturer

Date:

Signature

Name of Co-supervisor: Dr. Tan Suat Hian

Position: Senior Lecturer

Date:

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for the quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for the award of any other degree.

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Name : NURUL ASHIKIN BINTI ABDUL KARIM

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DEDICATION

Dedicated to my beloved parents

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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 condition makes it available in the market throughout the year. Phytochemicals is one of the important components in plant. 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 part; 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-Dichlorophenoxyaceticacid (2,4-D) 1.5 mg/L, 6-Benzylaminopurine (BAP) 0.5 mg/L with 2,4D 1.5 mg/L, Indole-3-Butyricacid (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 aeroginosa* (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 mgandungi kandungan TF paling tinggi. Ekstrak metanol kalus tersebut telah dikaji untuk aktiviti antibakteria dan antihypertensi.Tiga Gram-negatif: *Esherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC 1331) *Pseudomonas aeroginosa* (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 ektstrak kalus C3 dan ekstrak kalus C4 dapat membunuh bakteria berdasarkan diameter zon penyekatan dan kepekatan minima yang dapat membunuh bakteria. Kemudian, dalam kajian antihypertensi 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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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ANOVA	Analysis of variance
cm	Centimeter
B	6-Benzyladenine
B 5	B 5 medium
CE	Catechin hydrate
CFU	Colony forming unit
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
Fe	Iron
g	Gram
GA	Gallic acid
h	Hour
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
I	Indole-3-butyric acid
L	Litre
m	Metre
mg	Milligram
ml	Millilitre

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

℃	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.

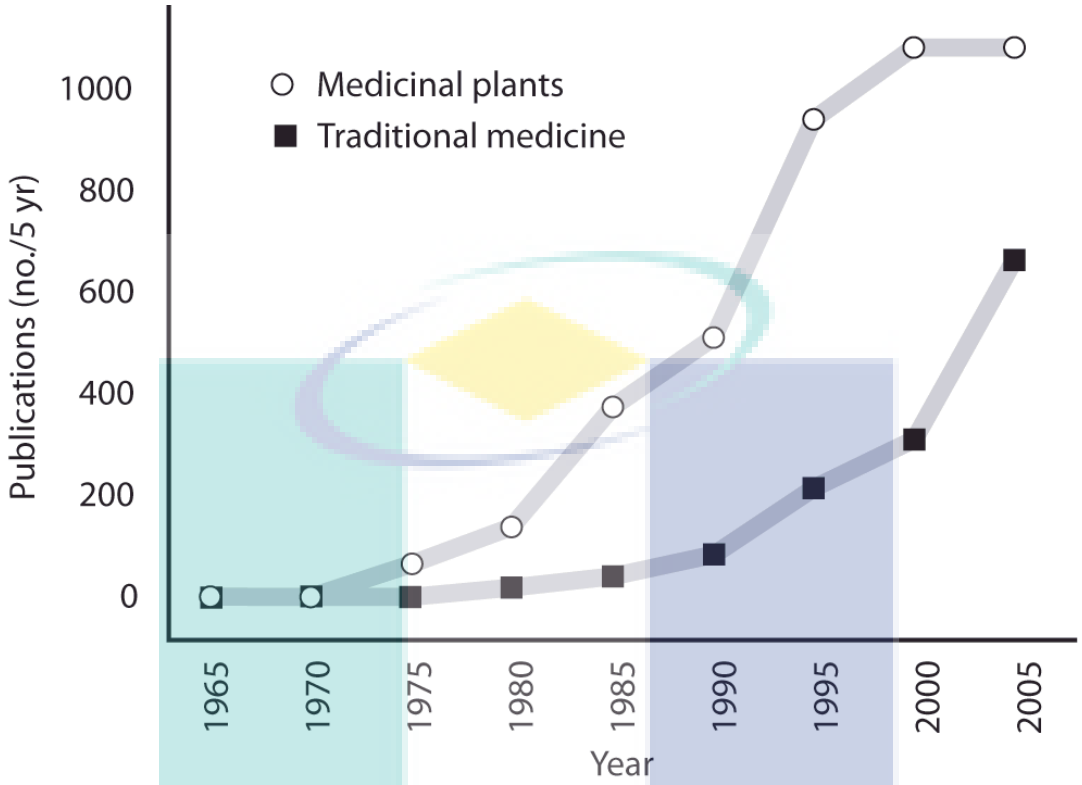


Figure 2.1: Graph showing the publications of medicinal plants and traditional medicine from 1965 - 2005 (Adapted from Janick and Whipkey, 2007).

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 hlp 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 cucrbits. 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 dietry 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 MUSKMELON

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 usuallu 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).

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).

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 B_6), nicotinamide (vitamin B_3), 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)

Chemical constituent	*Result*
Alkaloids	++
Triterpenoids	++
Flavonoids	+
Carbohydrates	+
Proteins	+
Phytosterol	+

++ = 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 bioactivites 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 a*l., 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.

The media often used in plant tissue culture are the important element in inducing the explant. There are medium without supplement of plant hormone and medium supplemented with plant hormone. These type of media will definitely induce different morphology towards the same type of explant. This is because plant hormone plays role in plant growth and regulation. But different types of explant may need different types of plant hormones at different optimal concentration for its growth regulation. Some explants may not need hormones to be induced.

2.4.2 Tissue culture of Muskmelon (*Cucumis melo L.)*

There are few published reports regarding the tissue culture of *Cucumis melo*. obtained were varied depending on plant growth regulators (PGRs) used, and laboratory. There are various plant parts used for culture of muskmelon, such as leaf, nodal segment and seeds. It also depends on the goal of the experiment whether to produce callus, plants or suspension culture.

Tissue culture of Muskmelon was done by previous researcher using proximal cotyledon as explant from variety the Gulfstream' and 'Charentais' U.S. The media for regeneration used in the study were as follows; MS salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al*., 1968), 100 mg *myo*-inositol/liter, 3% sucrose, and 5 μM benzylamino-purine (BAP) for 'Charentais' and 10 μM indole-3-acetic acid (IAA) and 5 μM BAP for 'Gulfstream'. The pH was adjusted to 5.8 with 1 N KOH or 1 N HCl and 0.7 % agar was added. The medium was autoclaved at 120 ºC at105 kPa for 15 min, and 5 ml was dispensed into 20-ml scintillation vials. Expanded green cotyledons were excised 2 mm beyond the point of attachment, transferred to the culture vials, and incubated at 25 °C.The findings for this experiment was that friable white callus was initiated around the cut edges of the cotyledon of 'Gulfstream'and 'Charentais' explant within 5 to 7 days in culture on their respective media. The callus differentiated into dark-green nodules, after transfer to MS + 0.5 μM BAP for 'Gulfstream' and to MS + 0.1 μM BAP for 'Charentais'. It produced leaves and shoots

within two to three weeks. In this study, it was stated that Muskmelon plants regenerated from cotyledons showed a high frequency of variation in morphology (Fassuliotis and Nelson, 1992).

In producing tetraploid melon, *in vitro* cultured cotyledon is the best source. In addition, microscopic study need to be done to confirm the stomata size, number of chloroplasts of guard cells, and pollen shape, thereby minimizing the need for absolute confirmation of ploidy level by chromosome counts.

There was a study done by Chan and Lok (2005) using nodal segment from Muskmelon variety 'London' as the explant. The medium used in this study was MS supplemented with BAP 0 - 10 mg/L. The findings of this study showed that MS supplemented with 2 until 10 mg/L of BAP induced all nodal segments produce multiple shoot (100%). It was reported that the best medium for multiple shoot production of melon was MS medium supplemented with 1 mg/L BAP with 0.2 mg/L NAA. But, some of the explants produced roots instead of shoots. In multiple shoot production in large numbers, they can easily produced using apical shoot derived from multiple shoot stock as the explant. With addition of 8 mg/L of BAP in the MS mediun, the explant produced callus. When the hormone added at 8 mg/L of BAP with 2 mg/L of NAA in the MS media, it induced the explant to produce callus.

2.4.3 Establishment of aseptic explant

Explants for *in vitro* plant propagation are usually derived from the outside environment, either from greenhouse or nursery. It is usually contaminated with microorganism and other sources of contaminants, so it has to be sterilized before it can be propagated. The microorganisms such as the bacteria or nematode have to be removed or killed in order to avoid explants contamination or killing of the plant. There are various sources of contamination in the cultures such as the soil and the dust on the plant (Dodds and Roberts, 1995). George and Sherington (1994) have stated that the

sterilization treatment may be different according to the season because microbial populations are dependent on the season.

Sterilizing agents are used in the procedures of obtaining aseptic tissues. Sodium hypochlorite (NaOCl) and calcium hypochlorite are commonly used as a sterilization agent as they are cheap and easily available. Sodium hypochlorite is available as commercial bleach, Clorox®. There are many studies shown that sodium hypochlorite is suitable for obtaining aseptic explants. Girija *et al.*, (1999) had successfully established aseptic explants using 0.5% (w/v) NaOCl for duration of 15 - 20 minutes. Other findings show that mercury chloride can also be used as a sterilizing agent.

2.4.4 Plant growth regulators

Plant growth regulators (PGRs) are a plant growth substances or plant hormone which are compounds that can be synthesized by the plant itself that involved in plant growth and regulation. They are very active at a very low concentration which is optimal for plant growth regulation. There are five main classes of PGRs, which are auxin, cytokinin, gibberellins, ethylene and abscisic acid (Walter, 2003). The most important and the most commonly used in tissue culture research are the auxin and cytokinin. Auxin is synthesized in the stem and root apices and it is transported along the plant axis (Lambers *et al.*, 2003). In plant regulation, auxin can promote both cell division and cell growth. It can also induce lateral root formation in stem cutting.

Cytokinin is synthesized in plant root and shoot. It is responsible to promote cell division, growth, development, delay senescence and act with auxin to control growth and development (Walter, 2003). The naturally occurring auxin and cytokinin are not available for routine use. The synthetic PGRs are extensively used in tissue culture

because of its stability. The most commonly used are 2,4- dichlorophenoxyacetic acid (2,4-D), 1-napthaleneacetic acid (NAA) and indole-3-butyric acid (IBA), Dicamba or picloram. Generally, when a high concentration of auxin and a low concentration of cytokinin are supplemented into the medium, they promote cell proliferation with the formation of callus. In the induction of root primordial, it can be initiated using auxin alone or combination of auxin with a low concentration of cytokinin (Pierik, 1997).

2.4.5 Callus culture

Callus culture is the maintenance and growth of the unorganized cell masses, which arise from the uncoordinated and disorganized growth of small pieces of plant or previously cultured cell. Callus is often used as a target tissue for genetic transformation. It is also utilized for isoflavonoid production such as in callus culture of *Prueraria tuberose*, the Indian kudzu (Romawat *et al.*, 2004). Plant cell culture is also important for the production of active source of biologically active compounds and attempts have been made to increase their accumulation (Romawat *et al.*, 2004). Verma and Jain (2011) stated that callus culture can be important for maintaining or protecting endangered plant species where it can be subcultured for many times. Different plant culture media may trigger different responses toward the callus induction.

There are various types of callus which varies depending on the parent tissue, age of the explant and the growth conditions. There are loosely packed callus cells, friable callus, lignified, and embryonic callus and coloured callus due to the presence of phytochemicals pigment.

In initiation and establishment of callus culture, there are four main important points to be followed. The first is the selection of suitable parent material. The explants must be grown vigorously and free from diseases as well as the explants must meet the desired genetic characteristics. Second, is the choice of explant and method of isolation.

The culture procedure must be done under aseptic condition, including the instruments. The explants must be decontaminated and surface sterilized. Next, the appropriate medium and culture condition must be provided to ensure optimal growth.

Finally the optimization of the culture condition. The most suitable medium for callus growth can only be obtained by trial and error. This is because different types of plant may need different medium composition for callus initiation (Yeoman, 1973). After the growth of callus, the growth must be monitored to observe the patterns of growth and differentiation. There are three stages of callus growth, which are induction of cell division, period of active cell division and slow down of cell division.

Plant callus culture is an efficient and alternative source for the production of biologically important secondary metabolites (Mulabagal and Tsay, 2004). Selection of cultured cell must be done meticulously to maintain high yield of secondary metabolites. The studies had been done in stimulation and biosynthetic activities of cultured cell using various methodologies in order to obtain high yields and concentration of secondary metabolites. Table 2.2 showed bioactive secondary metabolites from plant cell cultures which had been done previously for its active ingredients.

Table 2.2 Bioactive secondary metabolites from plant cell cultures

Plant Name	Active Ingredient	Culture Type	Reference
Agave amaniensis	Saponins	Callus	Andrijany et al., 1999.
Allium sativum L.	Allin	Callus	Malpathak and David 1986
Ambrosia tenuifolia	Altamisine	Callus	Goleniowski and Trippi 1999.
Bupleurum falcatum	Saikosaponins	Callus	Wang and Huang 1982.
Canavalia ensiformis L	Canavanine	Callus *Canavalia ensiformis* L	Ramirez et al., 1992.
Chrysanthemum cinerariaefolium	Pyrethrins	Callus	Rajasekaran et al., 1991.
Citrus sp.	Naringin Limonin	Callus	Barthe *et al.* 1987.
Corydalis ophiocarpa	Isoquinoline alkaloids	Callus	Iwasa and Takao 1982.
Duboisia leichhardtii	Tropane alkaloids	Callus	Yamada and Endo 1984.
Eriobotrya japonica	Triterpenes	Callus	Taniguchi *et al.*, 2002.
Eucalyptus tereticornis	Sterols and Phenolic compounds	Callus	Venkateswara *et al.*, 1986.
Glycyrrhiza echinata	Flavanoids	Callus	Ayabe *et al.*, 1986.

(Adapted from : Mulabangal and Tsay, 2004)

Papaver bracteatum	Thebaine	Callus	Day et al.1986.
Glycyrrhiza glabra var. *glandulifera*	Triterpenes	Callus	Ayabe et al., 1990.
Hyoscyamus niger alkaloids	Tropane	Callus	Yamada and Hashimoto 1982.
Mucuna pruriens	L-DOPA	Callus.	Brain K. R.1976.
Nandina domestica	Alkaloids	Callus	Ikuta and Itokawa 1988.
Nicotiana rustica C	Alkaloids	Callus	Tabata and Hiraoka 1976.
Nothapodytes foetida Callus Thengane et al.	Camptothecin	Callus	Thengane *et al*., 2003.
Ophiorrhiza pumila related	Camptothecin	Callus	Kitajima et al.1998.
Nothapodytes foetida.	Camptothecin	Callus	Thengane et al.2003.
Ophiorrhiza pumila	Camptothecin related alkaloids	Callus	Kitajima et al.1998.
Panax ginseng	Saponins and Sapogenins	Callus	Furuya et al.1973.

Table 2.2 Continued

Papaver somniferum L.	Alkaloids	Callus	Furuya et al.1972.
Rauvolfia serpentina x *Rhazya stricta* Hybrid plant	3-Oxo-rhazinilam	Callus	Gerasimenko et al., 2001.
Ruta sp.	Acridone and Furoquinoline alkaloids and coumarins	Callus	Baumert et al., 1992
Salvia fruticosa	Rosmarinic acid	Callus & cell suspension	Karam et al., 2003
Salvia miltiorrhiza	Lithospermic acid B acid and Rosmarinic	Callus	Morimoto et al., 1994.
Sapium sebiferum	Tannin.	Callus & cell suspension	Neera and Ishimaru, 1992
Scopolia parviflora	Alkaloids	Callus	Tabata et al., 1972.
Scutellaria columnae	Phenolics	Callus	Stojakowska and Kisiel, 1999
Tecoma sambucifolium	Phenylpropanoid glycosides	Callus	Pletsch et al.1993

Table 2.2 Continued

2.5 PHYTOCHEMICALS

Phytochemicals of plant are compounds that exist in plants which has no specific role in the basic life process (Parr, 1989) compare to primary metabolites, where it play an important role in the major activity such as photosynthesis, respiration, growth and development of plant (Ashihara *et al*., 2007).

It can be divided into three major groups, consisting of phenolics or polyphenolic compound terpenoids and alkaloids (Ashihara *et al*., 2007). It is important in the adaptation of plant to the environment; for protection against pests, as coloring, scent and plant hormones, and protecting from pathogens as well as preventing leaf damage from UV light (Bourgaud *et al*., 2001).

Important sources of active pharmaceuticals such as antibiotic and drugs can be found in plant secondary metabolites (Rao and Ravishankaar, 2002). There are 25 % of these substances used in cosmetic and nutraceutics (Bourgaud *et al*., 2001; Parr, 1989). In the study of methanolic extract of melon seed showed that it contained alkaloid, flavonoid, triterpenoid and phytosterol (Arora *et al*., 20011).

In order to provide for the correct identification of the plant species and to profile the chemical composition of the plants, HPLC analysis of the plant had been utilized in many studies to determine types of chemical compound present in the extracts (Springfield *et al*., 2005).

Phenolics can be detected by HPLC coupled with photo diode array detector with a high frequency accuracy. Markham (1982) described the two criteria used to determine the spectrum of flavonoid. The shape of UV spectra compared with the standard of UV spectra and wavelength of the peak. Ansari *et al*.,(2012) reported that in the phytochemical screening of fruit of *Cucumis melo* var. Namdhari, there was strongly

positive existence of phenolics and flavonoid compounds in the alcoholic extracts of the fruit.

2.5.1 Polyphenols

Polyphenols or known as phenolic derivatives of pentose phosphate, shikimate and phenylpropanoid pathways (Balasundram *et al*., 2006). It is one of the most important phytochemicals because of its health benefits. It can be characterized as compounds that have at least one aromatic ring with one or more hydroxyl groups attached. According to Caravaca *et al*.,(2006), about 8000 phenolic structures have been reported among plants.

Phenolics are usually present as conjugates with monosaccharide or polysaccharides that link to a phenolic group. It may also link to functional derivatives such as esters and methyl esters (Harborne, 1989). The characteristics of phenolics vary from simple, low molecular-weight, single aromatic ring compound to large and complex ones. Their number and arrangement of carbon atom can be used to classify these substances.

There are two major groups of phenolics, the flavonoids and the non-flavonoids (Ashihara *et al*., 2007). Phenolics can be divided into 10 types bases on structure; simple phenols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids and lignins (Caravaca ,2006).

Phenolic compounds are known to possess anti-carcinogenic, anti-inflammatory, anti-atherogenic, anti-thrombotic, immune modulating and analgesic activities and exert these functions as antioxidants (Vinson *et al*., 1998).

According to Bravo (1998), this compound carried important function in reproduction, growth, providing protection against pathogens and predators. Phenolics

can also determine color and sensory character of the plant (Alasalvar *et al*., 2001). Previous studies showed that the phenolics are involved in defense mechanism under extreme temperature, UV-B radiation and antioxidation and influenced on soil nutrient cycling.

Several methods developed to analyse polyphenols including colorimetric reactions, thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (Caravaca *et al*., 2006).

2.5.2 Flavonoid

Flavonoids are polyphenolic compounds composed of fifteen carbons with two aromatic rings linked by three carbon bridges (Cook and Samman, 1996). Figure 2.4 showed the major flavonoid structure generally found in plants. It can be classified according to the structures into major and minor structure with over 4000 different types.

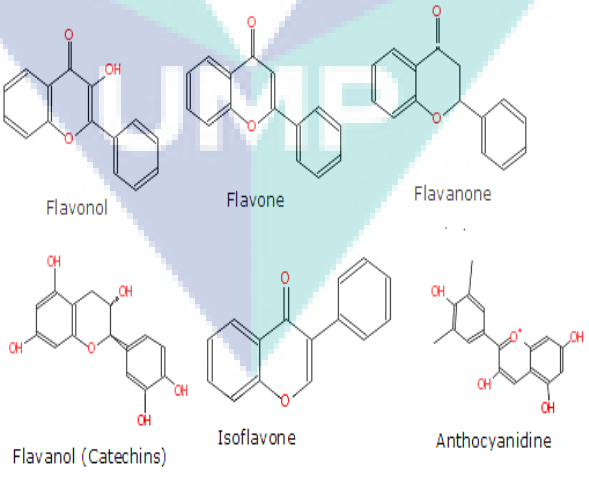


Figure 2.4: Major flavonoid structure generally found in plant.

Flavones, flavonols, flavonone, catechins, isoflavone and anthocyanidine are the major subclasses while for the minor subclass are dihydroflavonol, flavan-3,4-diol, coumarin, chalcone, dihydrochalcone and aurone (Ashihara *et al*., 2007).

It was reported that flavonoid can be found with various sizes of monomers and may be monomeric, dimeric and oligomeric. Tannin is one of the polymeric that can be classified according to structure which is condensed and hydrolyzed structure with gallic acid.

According to Ashihara *et al*.,(2007) sugars commonly attached to the majority of flavonoids exists naturally as glycoside. Biochemical activities and metabolites depend on the structure and orientation of molecule of the substances (Cook and Samman, 1996) in presence of sugar. Hydroxyl group may increase the ability of solubility while methyl group and isopentyl unit give flavonoid lipophilic ability.

There are various functions of flavonoid including UV protection, pigmentation, stimulation of nitrogen fixing nodules and disease resistance (Crozier *et al*., 2007). Plants increase the response for pollination and help other organism such as insect to find their food by production of colouration. Anthocyanin gives color to plant to attract pollinators (Coultate, 1990). Flavonoid is also known to possess antioxidant, free radical scavenger, and metal chelators and prevents any peroxidation on lipid (Acker *et al.*, 1996).

2.5.3 Flavones

It is reported that most flavones can be found in parsley, celery and some herbs with polymethoxylated flavones such as nobiletin and tangerine. It can be found in citrus species which is different compared to the flavonol that have a very similar structure (Ashihara *et al*., 2007).

According to Marten and Mithofer (2005), flavones are one of the largest subgroup of phenolic compounds. Luteolin and apigenin are two examples of flavones that have a- and c- ring substitution but do not have oxygenation activity at C3 that may have substitution such as methylation, hydroxylation, alkylation and glcosylation. Ashihara *et al.*, (2007) reported that this particular structure forms as 7-O-glycosides.

Flavones carry multifunction role that interacts with another organism and environment such as in ecology, agriculture, human nutrition and pharmacology and also in plant reproduction. Flavones are also involved in antioxidative activity that can help in preventing cancer cell and coronary heart disease, drug production and DNA recombinant activity (Marten and Mithofer, 2005).

2.6 ANTIBACTERIAL ACTIVITIES

A compound or substance that kills or slows down the growth of bacteria is said to have antibacterial activities (www.medterms.com). Antibacterial can be divided into two groups according to their speed of action and residue production.

The first group constitues substances that act rapidly to destroy bacteria, but quickly disappear by evaporation or breakdown and leave no active residue behind. Examples of this group are alcohols, chlorine, peroxides and aldehydes (Mohamad *et al*., 2012).The second group is a substance that mostly consists of compounds that leave long-acting residues towards the surface to be disinfected and have a prolonged action. Examples of this group are triclosan, triclocarban and benzalkonium (www.tufts.edu).

Many researches had been done recently to find new antibacterial compounds from plant extracts. There are many types of test for antibacterial activity such as disk diffusion assay, minimal inhibitory concentration (MIC) test and minimal bactericidal concentration test (Karsha and Lakshmi, 2010). The disk diffusion assay is common antibacterial test where the diameter of inhibition zone is used as a parameter to detect

antibacterial activity. Inhibition zone is the zone where bacteria did not grow on the agar plate due to the antibacterial agent activity. The bigger the inhibition zone the higher is the activity (Mahesh and Satish, 2008).

A 6 mm disk is used to put dissolved compound or plant extract to be tested on inoculated bacteria on the agar plate. Figure 2.5 shows Petri dish showing examples of disk diffusion assay (B) and zone of inhibition (A). Usually, the effectiveness of the antibacterial agent will be compared with negative and positive control (Karsha and Lakshmi, 2009). The positive control is the antibiotic and the negative control is usually the solvent that have been used to dissolve compound or plant extract. One agar plate can be used for five different compounds or triplicate of compounds to be tested to according to the size of plate. The bacteria with optimal growth condition will be used in the experiment.

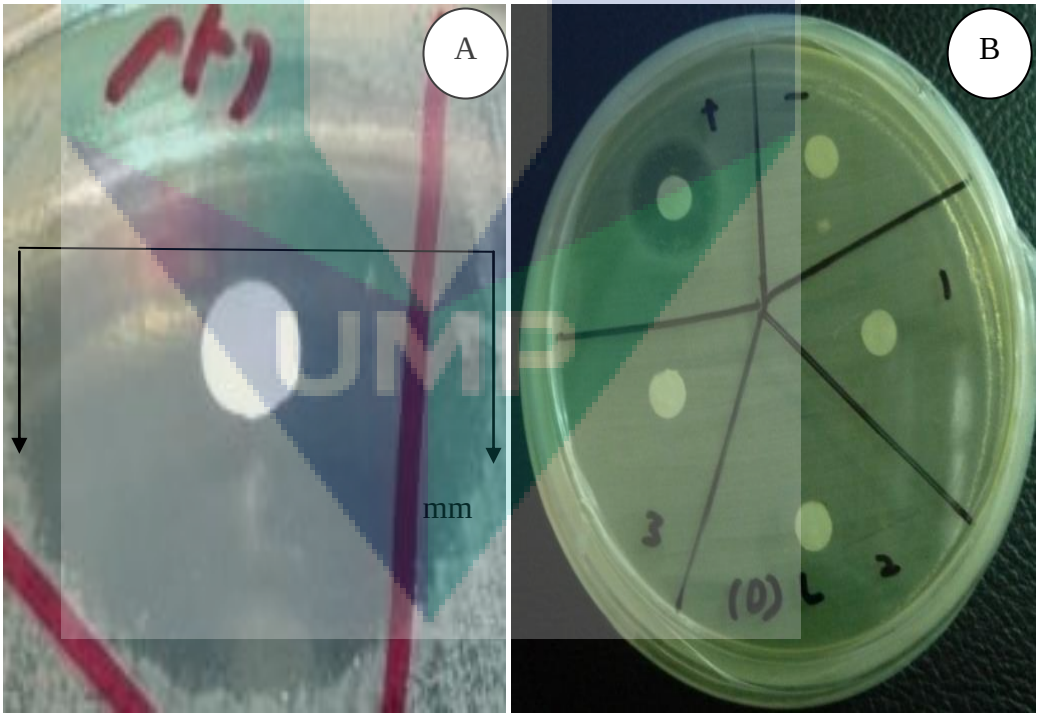


Figure 2.5: Petri dish showing disk diffusion assay (B) and zone of inhibition (A)

Minimal inhibitory concentration (MIC) is a test to find the lowest concentration of a compound or extract that can inhibit the growth of the tested bacteria and 96-wells plate has been used for the test.

Figure 2.6 showed a typical 96-well plate. The final volume for each well can be up to 200 µl. A known concentration of bacteria with plant extract or controls will be pipetted in the plate and incubated. The turbidity of the solution has been measured as the antibacterial activity. The lower turbidity indicates the compounds is successful in inhibiting the growth of the bacteria (Eloff, 1998).

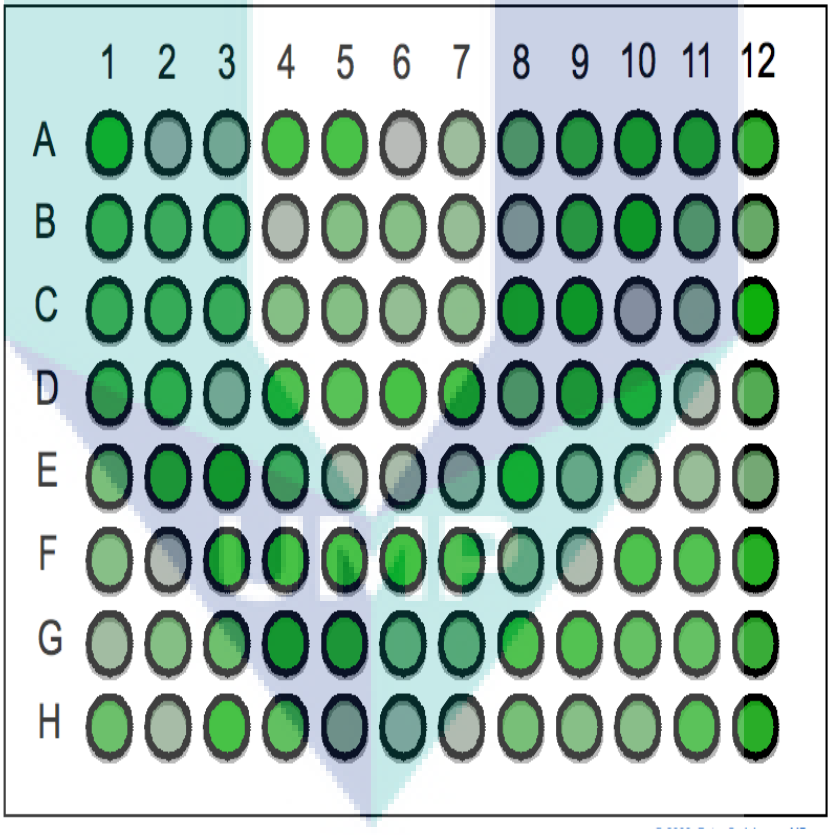


Figure 2.6: Typical 96-well plate

2.7 ANTI – HYPERTENSIVE ACTIVITY

Hypertension is the medical term for high blood pressure. High blood pressure can be either as primary or secondary stage. Primary or stage one high blood pressure occurs when the blood pressure is within 140 – 159 (mmHg) for systolic and 90 – 99 (mmHg) for diastolic. When the blood pressure is consistently over 160 (mmHg) for systolic and 100 (mmHg) for diastolic, it is considered secondary or stage two high blood pressure (Foex, 2004).

Normal blood pressure for the average adult human is around 120 (mmHg) for systolic and around 80 (mmHg) for diastolic (Foex, 2004). Many people did not realize when they have high blood pressure until it become serious.This is because high blood pressure has no initial symptoms. However, it can lead to long term diseases as well as complications such as organ failure, heart attack, stroke, peripheral artery disease and weakening the wall of the aorta (Robertson, 1987).

Hypertension can be treated with medicine or lifestyle adjustment in diet and exercises. There are various medicines provide for treatment of high blood pressure which is called antihypertensive drug. Antihypertensive drug is a class of drug that is used to treat hypertension. There are many types of anti-hypertensive drug presented in the market (Calhoun *et al*., 2008).

But, the healthcare providers recommended herbal and supplemental treatment or lifestyle changing in order to treat hypertension as drug therapies may lead to other health issues. A recent study reported that hibiscus tea is effective in lowering blood pressure (Diane *et al*., 2010).

ACE inhibitors work to inhibit angiotensin I from becoming Angiotensin II thus can avoid vasoconstriction that caused an increased blood pressure and release of aldosterone. Figure 2.7 showed the mechanism of action and side-effects of angiotensin

converting enzyme (ACE) inhibitors. The release of aldosterone leads to sodium and water retention in the blood. ACE is a non specific enzyme that can catalyse the vasodilator such as kinin (Nancy *et al.*, 2013).

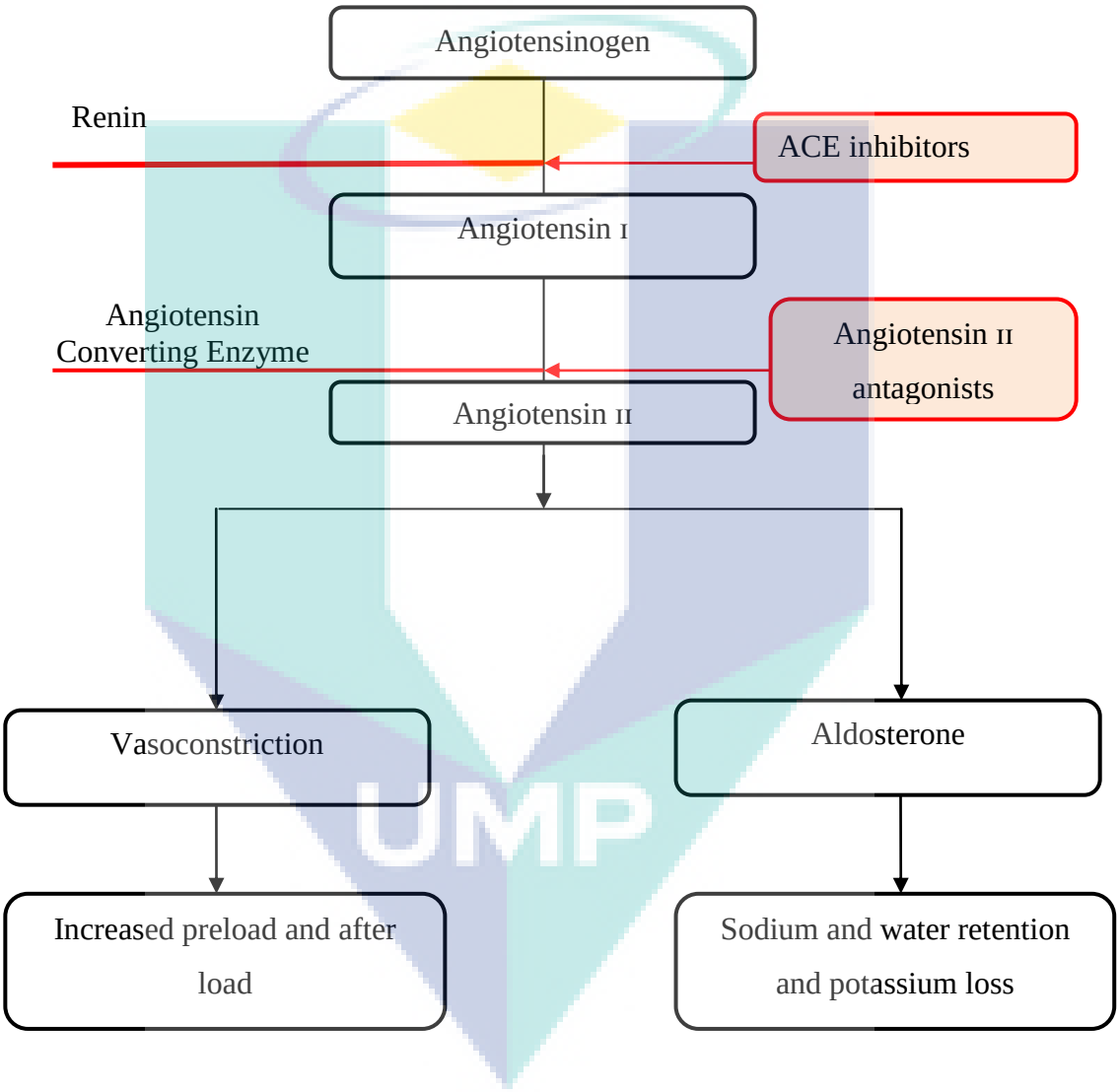


Figure 2.7: The mechanism of action and side-effects of angiotensin converting enzyme (ACE) inhibitors

Captopril and other ACE inhibitors are competitive inhibitors of ACE, that mimicking the structure of its substrate. Captopril is one of the active molecules. Usually, to become function in the mechanism, other drug need to be converted to active metabolites.The formation of Angiotensin –II will be directly block by ACE inhibitors, which at the same time increase bradykinin level. As a result, vasoconstriction are reduced, as well as sodium and water retension, and increased vasodilation (through bradykinin) (Burt *et al*., 1995).

ACE inhibitors are primarily used when the first-line diuretics or β-blockers are ineffectively working to lower the blood pressure. Usually, ACE inhibitors are most effective in young hypertensive patients. But when ACE inhibitors are used together with diuretics, the effectiveness will be different. ACE inhibitors are also working better in patients with higher renin level. Commonly used in patients following myocardial infarction, and in patients with chronic congestive heart failure. Synthetic ACE inhibitors usually have many adverse effects and toxicity and can not be consumed by pregnant woman. There are good drugs available in the market for ACE inhibitors such as Captopril, Rampiril and Enalpiril (Quan, 2006), but due to the adverse affect and toxicity, research continues to investigate natural ACE inhibitors which could be found in food sources.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 SELECTION OF PLANT MATERIAL FOR CALLUS INDUCTION

Three plant parts were taken, leaf, stem and fruit explants selection by extracting the secondary metabolites of plant parts extracts. All the plant parts were weighted and extracted for quantification of total phenolics (TPC) and total flavonoids content (TFC). The plant part with highest total phenolics and total flavonoids content were chosen as explants for callus induction. Figure 3.1 shows a summary of research activities starting with explant selection, callus induction and maintenance, bioactivities and final data analysis.

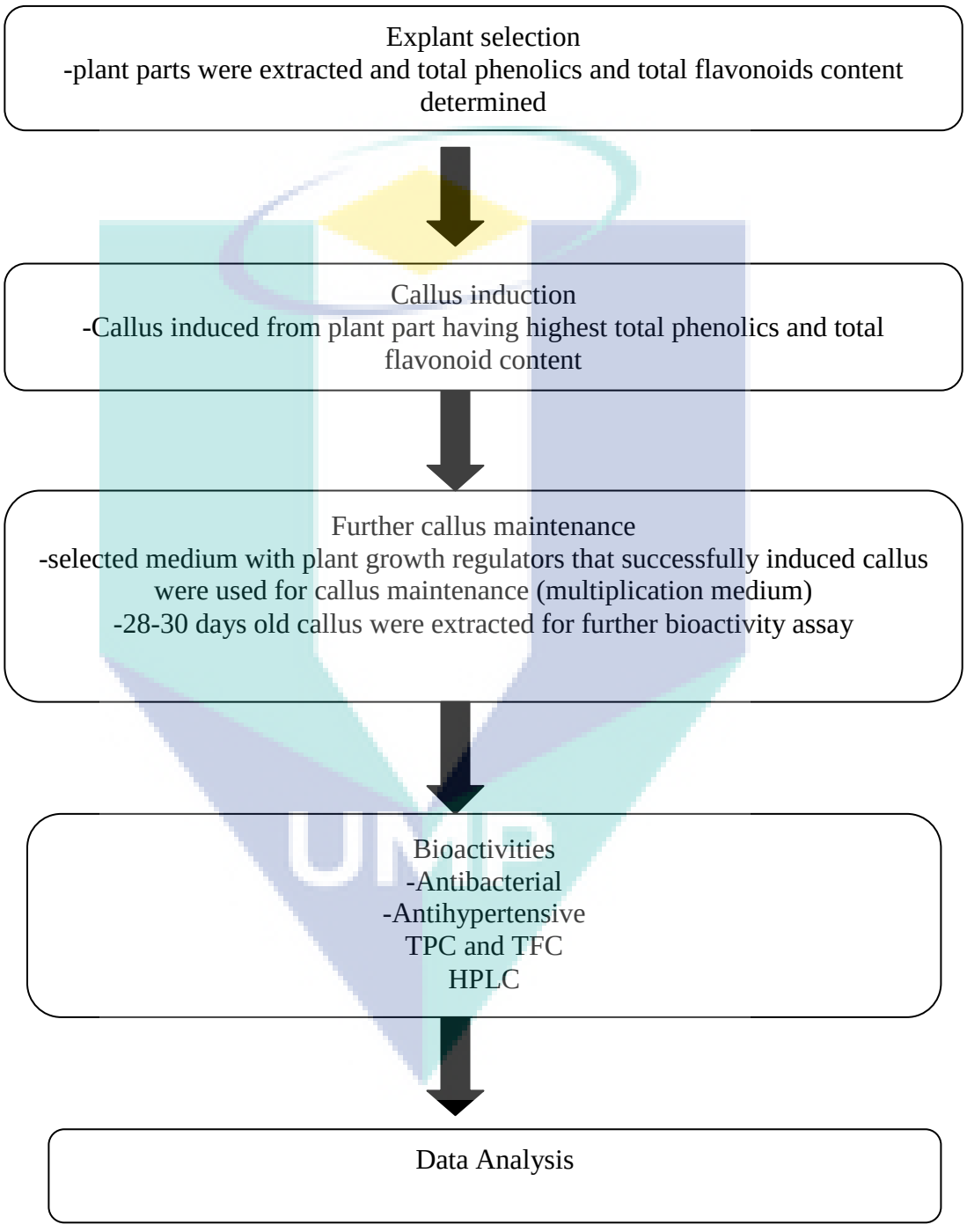


Figure 3.1 : Flow chart of research activities

3.2 PREPARATION OF STOCK SOLUTION

A known amount of auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D) and indole-3-butyric acid (IBA) was dissolved in 1 ml of alcohol and made up to the final volume, 100 mL with distilled water. The 6-benzyladenine (BAP) and Zeatin was dissolved in 1 ml of 0.1 N HCl or NaOH and prior added with distilled water to 100 mL final volume. The entire phytohormone stock solution was adjusted to pH 5 and stored in 4°C. MS basic (Murashige and Skoog, 1962) medium with vitamin B5 (Gamborg *et al*., 1968) was used in this experiment. The stock solutions were prepared according to lab manual provided. All plant growth regulators (PGRs) used in this study were purchased from Merck.

3.3 SURFACE STERILIZATION OF PLANT MATERIAL

The muskmelon seeds were obtained from a supplier in Terengganu (GM Peladang). It was germinated in the lab and the sterilization of the seed was done before germination. The seeds were left under running tap water added with soap for 15 minutes. Double sterilization techniques were used for the seed culture. In the laminar flow, the seed was immersed with 70 % ethanol for 2 minutes. Next, the seeds were rinsed with autoclaved distilled water and the water was decanted. Two drops of tween 20 (Sigma) were added as a wetting agent into 20 % Clorox ® with the seeds and the seeds were shaken constantly for 20 minutes. After the Clorox ® and the tween 20 were decanted, the seeds were rinsed again with sterile distilled water for five times. Next, the Clorox ® and the tween 20 were added in once again and the explants were constantly shaking for 15 minutes. The Clorox ® mixtures were decanted and the explants were rinsed again for five times with sterile distilled water and the explants were put on the MS medium.

Once it was germinated for ten days, the leaf was taken as explant. The leaf was left under running tap water for about 10 - 15 minutes to avoid the first stage of

contamination. Then, the explant was put under the laminar flow chamber for about 20 minutes before further sterilization process. Next, the explants were rinsed and shaken with 70 % ethanol in the first sterilization process for three 1 minute. Then, the explants were rinsed with sterile distilled water. After that, 10 % (v/v) Clorox ® bleach with a few drops of tween 20 were used for the final sterilization process. The explants were immersed in the solution for 10 to 15 minutes, followed by rinsing for five times with sterile distilled water. Tween 20 was used as a wetting agent and can enhance the work of the bleach so the leaf can be sterilized within a short time without damaging the plant tissue.

3.4 MEDIA TREATMENT AND CULTURE CONDITION

The dead tissues of calli were excised prior to transfer onto treatment media (Table 3.1). Two auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-butyric acid (IBA) and two cytokinin hormones, 6-benzyladenine (BAP) and Zeatin were used in this study. 6-benzyladenine (BAP), Zeatin and indole-3-butyric acid (IBA) at 0 - 2 mg/L was combined with 2,4-dichlorophenoxyacetic acid (2,4-D) at 0 - 2 mg/L, respectively. Sucrose (Fischer) was supplied at 30 g/L, and 2.5 g/L agar (Phytotechnology) was used to solidify the media. The medium was adjusted to pH 5.7 ± 0.1 with 0.1 N HCl or 0.1 M NaOH solution. Ten ml of medium was distributed into flasks. Media were sterilized by autoclaving (Hiclave HVE-85, Hirayama) at 106 kPa, 121°C for 15 min. All cultures were incubated 25 °C with photoperiod 16 h daylight with white fluorescent lamp (Panasonic) at 31000 Lux.

3.5 CALLUS INDUCTION

The excised part of the leaf, (1 × 1 cm) was used as explants for callus induction. The small excised leaf pieces were transferred onto the surface of agar solidified MS media, supplemented with plant growth regulators (PGRs). The media were placed in sterile vials and left in the plant tissue culture room that was equipped

with a 16 h light with white fluorescent lamp (Panasonic) at 1000 Lux and 8 h dark conditions. Every 3 to 4 days, the monitoring of callus was done to check on callus formation and any microbial infections.

The callus initiation and productions were observed. Calli were separated from explants after up to 3 weeks of initiation and were transferred to a fresh solid multiplication media. Calli were extracted for phenolics and flavonoids content every week up to the fifth week as well as its weight were determined. Biomass of calli were maintained under aseptic condition until for further experiment.

3.6 PREPARATION OF COMPOUND

The modified method of Richard *et al*., (2011) was used for the extraction and quantification of the phytochemicals of the callus. The callus from each selected medium was ground with mortar and pestle. Then, 70 % of methanol was added by small volume until the mixture were homogenized. The methanol was added to make up the volume to 10 ml and the slurry was shaken at 225 rpm using orbital shaker (ERLA, E-TS-585) for about 72 h and then filtered. Then, the solvent was evaporated using a rotary evaporator. After that, the extracts were weight to get dry volume (1 g).Then the extracts were re-dissolved with 10 ml methanol and were used for bioactivity test.

3.7 PHYTOCHEMICALS CHARACTERIZATION OF CALLUS EXTRACT

3.7.1 Total phenolics content (TPC)

The folin-ciocalteu method by Waterman and Mole (1994), and Woisky and Salatino (1998) with slight modification were used to measure total phenolics content of callus extract in the experiment. Extract solution of callus (1 ml) was added into a 50 ml volumetric flask. Four ml of folin-ciocalteu reagent (Sigma) were added to it. Six ml of 20 % (w/v) sodium carbonate solution and 15 ml of distilled water were added into the solution. Then, the final volume of the solution was made up to 50 ml with distilled water. The solution was incubated at room temperature for 2 h. After 2 h of incubation, the absorbance was measured using UV-Vis spectrometry (LIBRA) at 760 nm. Concentration of gallic acid were calculated using standard curve of gallic acid as constructed with a concentration ranges from 0-800 µg/ml (M. Lopez-velez *et al*., 2010). Total phenolic content were estimated using equation (1) . The assay was carried out in triplicate.

Total phenolics content: $\frac{G \times V \times df}{M}$ (1)

Where:

G: Gallic acid concentration determined from standard curve (mg/ml)

V: Volume of solution used (ml)

df: Dilution factor

M: Sample weight (g)

3.7.2 Total flavonoids content (TFC)

The aluminium chloride complex formation method as suggested by Bonvehi and Coll, (1994) and Popova *et al*., (2003) were used to measure the flavonoid content of callus extract with slight modification. A 20 ml methanol and 5 % Al_2Cl_3 (w/v) were

added in 2 ml of the extract solution. The solution was made up to 50 ml with methanol. Then the mixture was left for 30 minutes at room temperature.

After that, absorbance was measured using UV-Vis double beam spectrometry (LIBRA) at 425 nm. The assay was carried out in triplicates and methanol was used as blank. Concentration of catechin hydrate was estimated using a calibration curve of the catechin hydrate at a range of 0-200 mg/ml (Popova *et al*., 2005) and total flavonoid content were derived using equation (2).

Total flavonoid content: $\frac{C \times V \times Df}{M}$ (2)

C: Concentration of catechin hydrate (mg/ml)

V: Total volume of solution (ml)

Df: Dilution factor

M: Sample weight (g)

3.8 ANTIBACTERIAL TEST

Antibacterial activity test was performed according to the modified method reported by Kirby and Bauer (1966).

3.8.1 Preparation of bacteria for treatment

Three Gram-positive and three Gram negative bacteria were used in this study. *Staphyloccus aureus* (ATCC baa1026), *Pseudomonas aeroginosa* (ATCC 15442), *Bacillus subtilis* (ATCC 1774), *Escherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC 1331) and *Enterococcus faecalis* (ATCC 14506). The bacterial strains were obtained from the Faculty of Industrial Sciences and Technology and subcultured and incubated before use. Streptomycin was used as positive control and the solvent was used as negative control.

The selected bacterial strains were grown on nutrient agar, and the agar plates were placed in an incubator at 37 ˚C overnight or about 18-24 h before it can be used for the antibacterial test. A 0.85 % (w/v) of the saline solution was used to suspend the bacteria or it can be done using nutrient broth (NB). The turbidity values of the bacteria solution were measured using a spectrophotometer at 600 nm to get the required reading. The reading must be obtained to a turbidity value of Mac Farland standards (10^8 CFU/ml). The suspensions were then diluted in order to get the inocula for treatment in Muller Hinton Broth to give 10^6 colony forming units (CFU/ml) (ranges 0.08- 0.132) (Ezoubiri *et al*., 2005).

3.8.3 Disk preparation

A known concentration of callus extracts was sterilized through 13 mm manual syringes completed with 0.45 µm syringe filter. 6 mm disks were prepared and sterilized. Then, the 6 mm sterile disks were impregnated with 10 µl of sterile extracts. A 70 % ethanol was diffused to disk to be used as a negative control. Commercially available antibiotic diffusion disk were used for comparison.

3.8.4 Disk – diffusion assay

Twenty ml of sterile Mueller Hinton agar (Difco Becton Dickinson, USA) were poured into sterile petri plates. Then, the inocula were spread on top of the solidified media. The agars were let to dry for 10 min at room temperature. After that, the disks that have been impregnated with callus extracts, positive and negative control were applied onto the agar and left for 30 min at room temperature. It is in order to let the extracts and the controls to diffuse into the agar before incubation for 24 h at 37 °C inside the incubator. The inhibition zone were measured in millimeters. The extracts inducing inhibition zone more than 3 mm around the disc were considered as antibacterial agent (Rabe and Van Stadten., 1997). The tests were performed in triplicate.

3.8.5 Minimal inhibitory concentration (MIC)

The antibacterial activity was investigated by using a modified micro dilution method in culture broth, recommended by Ellof (1998) and the National Committee for Clinical Laboratory Standard (NCCLS, 2001). The bacterial strains were activated in nutrient broth BHI, (Difco) for 24 h at 37 ˚C. 100 µl of Mueller Hinton broth were transferred into 96-wells plate. The final volume of each well was 200 µl. Each well was filled with 100 µl culture medium, 90 µl callus extract and 10 µl of suitable bacteria inoculum. Antibiotic was used for control. All experiments were performed in triplicate and the 96-wells plates were incubated at 37 ˚C for 24 h. Antibacterial activity was detected using visual observation and turbidity values measured by micro plate reader. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of extract able to inhibit the bacteria growth (turbid or clear).

3.9 ANTIHYPERTENSIVE TEST USING ACE (ANGIOTENSIN-CONVERTING ENZYME) KIT

The antihypertensive test was done using a general protocol of ACE kit using a mictrotiter plate. A 20 µl portion of sample solution were added to each well and deionised water as blank to each well. Twenty µl of buffer were added followed by 20 µl of deionised water to each well. Then, 20 µl of enzyme, working solution were added to each sample and blank well and the plate was incubated at 37 °C for 60 min. Each well was added with 200 µl of indicator working solution. The plate was incubated at room temperature for 10 min. The absorbance value was read at 450 nm with a micro plate reader.

The ACE inhibitor activity (inhibition rate %) was calculated using the equation:

ACE inhibitory activity (inhibition rate %) = $[(A_{\text{blank 1}} - A_{\text{sample}}) / (A_{\text{blank 1}} - A_{\text{blank 2}})]$ X 100

Blank 1: positive control (without ACE inhibition), blank 2: reagent blank

Table 3.1: Composition of the reaction mixture for antihypertensive test

Reagent	Sample	Blank 1	Blank 2
Sample solution	20µl	-	-
Deionized water	-	20µl	40µl
Substrate Buffer	20µl	20µl	20µl
Enzyme working solution	20µl	20µl	-
Indicator working solution	200µl	200µl	200µl

3.10 CHEMICAL PROFILING BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC was conducted on a Hewlett–Packard (HP) 10980 Liquid Chromatography. It was fitted with a C-18 reverse phase (5µm) column (25 cm × 4 mm LD) Latex Eppelheim, Germany. As for the individual separation of compounds in the extract, acetic acid in water (2 % v/v) was used as eluent A and methanol was used for seluent B in mobile phase and 20 µl of the extract was injected.

Polyphenols were eluted by a consisted of 95 % A for 2 min, 75 % A for 8 min, 60 % A for 10 min, and 0 % A until completion of the run by 45 min. The mobile phase's flow rate was maintained at 1 ml/min and the phenolic compounds in the elute were detected with a UV diode-array detector (HP 1040M) set at 278 and 340 nm. Instrument control and data handling was by means of a HP chemstation operating in the Microsoft windows software environment. Commercial gallic acid (GA) and catechin hydrate (CE) were use as standard.

3.11 STATISTICAL ANALYSIS

The callus was induced by the selected MS media with PGRs.The sample was harvested and ground using mortat and pestle. One gram of each callus was extracted as described in Chapter 3. The antibacterial and antihypertensive properties of the extracts were analysed. The total phenolics and total flavonoids were calculated the contents of gallic acid and catechin hydrate were analysed by HPLC. Each analysis was repeated three times. The data were compared by T-Test, one-way analysis of variance (ANOVA) followed by Tukey test to find differences between treatment means at 95 % ($p<0.05$) significance level of the sample and correlation test.Statistical analysis was done using SPSS software for Windows computer statistic programme.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 SELECTION OF PLANT MATERIAL FOR CALLUS INDUCTION

The part of plant to be selected as an explant in callus induction is important in order to meet the goal of the study. Therefore, to choose the part of the plant with the highest total phenolics and total flavonoids content, the screening must be done to determine the suitable part of the plant. Table 4.1 shows the data for total phenolics content (TPC) and total flavonoids content (TFC) for methanolic extracts of different plant parts from *Cucumis melo* L.

Interestingly, the leaf shows the highest value for both total phenolics and total flavonoids content which is 110.80±0.06 mg GAE /g and 126.71±0.06 mg CE /g. The fruit shows the lowest value of total phenolics and total flavonoids content which are 14 ±0.578 mg GAE /g and 20.43±0.08 mg CE /g. This result showed a significant difference in the total phenolics and total flavonoids contents among the extracts.

Table 4.1: Total phenolics content (TPC) and total flavonoids content (TFC) of methanolic extracts in different plant part.

Plant Part	TPC (mg GAE/g)	TFC (mg CE/g)
Fruit	14.00±0.57	20.43±0.08
Stem	20.00±0.57	35.57±0.01
Leaf	110.80±0.06	126.71±0.06

Values are mean ± standard deviation of triplicates (n= 3)

According to the result in selecting plant material (table 4.1), the leaf contained the highest amount of both total phenolics content and total flavonoid content. This indicated that the leaf was the best plant part to be used for callus induction in this study. Methanolic extract usually shows high total phenolics and total flavonoid content (Arora *et al*, 2011). In the study by Samatha *et al*., (2012), they stated that methanolic seed extracts of *Roxylum indicum* L. showed high accumulation of total phenolics content while methanolic extract of bark of the *Roxylum indicum* L. shows the highest accumulation of total flavonoid content compared with the extract with other solvents.

The highest content of total phenolics and total flavonoid were detected in the leaf extract, whereas the lowest content was measured in the flesh extract of *Roxylum indicum*. There are many factors that resulted in different accumulation of total phenolics and total flavonoids content in different part of the plant. Light intensities affect the secondary metabolites production in three varieties of L*ubisia pumila* B. (Karimi *et al*., 2013). This suggested that plant that grown naturally could cause different light intensity received by different part of plants.

Leaf usually receives high light intensity due to its surface area, size and shape. *Cucumis melo* is a climbing plant, therefore, light intensity was higher in leaf followed by its stem and fruit because the leaves size of the plant were mostly bigger than its stem. Thus, the accumulation of secondary metabolites was higher in the leaf part of *Cucumis melo* L. plant. In the biosynthesis of phenolics acid, phenylalanine

ammonialyase (PAL) is an important enzyme for production of phenolics acid. The enzyme activity is specifically induced by light intensity (Kumari *et al*., 2009).

Therefore, high production of phenolic acid in the plant part could be related to PAL enzyme activity. Total phenolics content of leaf extract was higher than other parts of the plant. It had been reported from previous studies for the plant of *Petroselinum crispum, Coriandrum sativum* and *Beta vulgaris*, (Pyo *et al.*, 2004; Wong and Kitts, 2006).

This suggests that in most plant, the leaf possessed high total phenolics content. The result is in good agreement with previous result reported by Hajar *et al*., (2009), where total phenolics of *Cucumis melo* methanolic extract of leaf was shown to be the highest. As conclusion, the leaf of the *Cucumis melo* is the most suitable explant for callus induction in this study.

4.2 PLANT GROWTH REGULATORS FOR CALLUS INDUCTION FROM *Cucumis melo* L. LEAF.

Based on the result for the highest total phenolics and total flavonoids content, the leaf part was used as the explants for callus induction. Apart from explant selection, plant growth regulators (PGRs) played important roles in the growth of the culture. Table 4.2 shows callus induction of melon leaf explants using MS medium added with different plant growth regulators (PGRs). The result, indicated that MS media supplemented with 2,4-D and IBA alone can induced callus without addition of cytokinin.

Table 4.2, MS media supplemented with hormone 2,4-D with the concentrations ranging from 0.5 - 1.5 mg/L mostly could induce callus with successful rate. There were 28.3 % induction at 0.5 mg/L, 68.3 % at 1.0 mg/L and 88 % at 1.5 mg/L of 2,4-D. MS media supplemented with BAP only produce callus at the edge of the leaves of the explant without further response. However, MS media supplemented with 1.5 mg/L

zeatin did not show any response of growth. This result showed a significant different in the induction of callus.

Table 4.2: Effect of different plant growth regulators (PGRs) on callus induction from muskmelon (*Cucumis melo* L.) leaf explants after 4 weeks of culture on MS media.

PGRs	Concentration (mg/L)	Induction rate (%±SD)
Control	0.0	-
2,4-D	0.5	28.3±0.45
2,4-D	1.0	68.3±0.47
2,4-D	1.5	88.0±0.32
IBA	0.5	-
IBA	1.0	-
IBA	1.5	65.0±0.48
IBA + 2,4-D	1.5 + 1.5	-
Zeatin	1.5	-
Zeatin + 2,4-D	1.5 + 1.5	80.0±0.41
BAP	1.5	-
BAP + 2,4-D	1.5 + 1.5	86.7±0.34

*Experiment carried out with at least 10 replicates /treatment
- : No induction or further response occurred

MS media supplemented with IBA ranging from 0.5 to 1.5 mg/L showed diverse results. At concentration of 0.5 to 1.0 mg/L of IBA, there was a growth of root (Figure 4.1 and 4.2) while at concentration 1.5 mg/L, it showed callus induction rate at 65 %. 2,4-D at 1.5 mg/L shows the highest induction rate among single PGRs because it is a good and strong auxin. In the result, the induction rate increased as concentration of the PGRs increased. Inclusion of an auxin can be necessary for callus growth and higher auxin concentration may require for callus induction (Abu-Romman *et al.,* 2013). The result of MS media supplemented with IBA were in agreement with the statement as well. This is because at high concentration of IBA (1.5 mg/L) in the MS media, it was able to induce 65 % callus. However, at a lower concentration of auxin in the media, it induced root formation. Auxin usually responsible for wound response, promoting differencial growth and many development process (Heisler *et al.*, 2005). This result

suggests that auxin was mostly suitable for callus formation from leaf of *C.melo* L. which MS media supplemented with 1.5 mg/L of 2,4-D gave the best result.

MS media supplemented with cytokinins, Zeatin and BAP also shows different result. There was no callus induced in MS media supplemented with 1.5 mg/L of Zeatin. Meanwhile, MS media supplemented with 1.5 mg/L of BAP showed callus induced only at the edge of the leaf of the explant and the growth of callus was slow after subculture. This showed that MS media supplemented with single treatment of cytokinins were not suitable for the callus formation from leaf of *C. melo*.. MS media supplemented with Zeatin only showed the explant swell in their size without further response after fourth weeks.

In contrast, the results for the combination of cytokinin and auxin in MS media showed better results. MS media supplemented with both 1.5 mg/L BAP and 2,4-D 1.5 mg/L gave the highest induction rate for combination of PGRs, which is 86.7 % following MS media supplemented with 1.5 mg/L of both Zeatin and 2,4-D with 80 % induction rate. These indicated that combination of BAP and 2,4-D was a good PGRs combination for callus induction of leaf from *C. melo.* This finding was in agreement with plant growth regulators interaction of auxin and cytokinin, that the same concentration of auxin and cytokinin would leads to callus induction (Martin *et al.*, 2010).

This also suggested that different plant have different adaptation when producing callus with plant growth regulators combination. Kim *et al.*, (1997) and Custurs *et al*., (1990) reported that the calli was able to induce using medium containing 2,4-D combined with BAP using explant derived from cotyledon and leaf of Muskmelon. It is reported by Oridate and Oosawa (1986) that callus could be induced after 21 days on MS supplemented with 4.5 µM 2,4-D and 0.4 µM BAP, 0.5 µM 2,4-D and 0.4 µM BAP using explants from cotyledon and hypocotyls from *Cucumis melo*.

In the study reported by Tabei (1991) and Molina (1995), they stated that the concentration of plant growth regulators for callus induction in melon plant was 0.5 µM 2,4-D combined with 0.4 µM BAP using explant from petioles, hypocotyls, leaf and cotyledon. In a condition of high ratio of auxin over cytokinin, high possibility of embryogenesis, secured callus initiation and root initiation while high ratio of cytokinin to auxin, auxiliary and shoot proliferation produced (Davies, 2004). This evidence supports the results that show effect of plant growth regulators due to the presence of auxin and cytokinin.

MS media supplemented with the auxins, in combination of 1.5 mg/L 2,4-D and 1.5 mg/L IBA showed callus formation at the edge of explant but without further response after 28 days. It is showed that the PGRs combination of the two auxins was not good for callus induction, as the concentration for the callus formation could be too strong or too much. Although auxin usually can promote callus growth but according to previous study done by Wernick and Milkowitz (1984) stated that auxin could show herbicide-like properties which can inhibit the callus formation at high concentration.

Callus morphology obtained from culture in MS supplemented with 0.5 mg/L and 1.5 mg/L 2,4-D were friable and yellowish in colour as observed in leaf derived callus (Figure 4.2 G and 4.3 I). The callus physical appearance from culture in MS supplemented with 1.0 mg/L 2,4-D was loose and greenish (Figure 4.2 H). Observation showed that the explants cultured in MS media supplemented with 1.5 mg/L IBA produced generally friable and yellowish callus in colour (Figure 4.2 F). Callus morphology obtained from culture in MS media supplemented with 1.5 mg/L Zeatin with 1.5 mg/L 2,4-D were friable and yellowish in colour (Figure 4.3, J). The callus observed from culture in MS supplemented with 1.5 mg/L BAP with 1.5 mg/L 2,4-D were friable and greenish in colour (Figure 4.3 K).

One of the functions of plant growth regulators is to coordinate growth and development of plant. According to Razdan (2003), the ratio of auxin and cytokinin is important to determine the morphology in culture. This occurs due to effect of sensitivity or responsiveness of plant to multiple hormones. The interaction between hormones gives various levels of responses.

The effect of one hormone may due to the help of another hormone because they may work in synergism (Davies, 2004). Karimi *et al*., (2012) also stated that one of the most important things to consider for callus induction is plant growth regulators. Thus, the results supported that different PGRs gave different responses toward callus induction.

The result from above experiment indicated that 2,4-D and IBA at 1.5 mg/L, 1.5 mg/L Zeatin with 2,4-D 1.5 mg/L and 1.5 mg/L BAP with 2,4-D 1.5 mg/L were suitable for callus induction of leaf of *Cucumis melo* L. However, 2,4-D was the best auxin for callus induction of the leaf of *Cucumis melo* L. studied. MS medium alone failed to induce callus, even cultures were kept for prolonged period.

As a conclusion, the best single treatment for callus induction was MS media treated with 1.5 mg/L 2,4-D and the best combination treatment was MS media treated with 1.5 mg/L BAP and 1.5 mg/L 2,4-D. The selected concentrations of plant growth regulators (PGRs) for callus induction of Muskmelon (*Cucumis melo* L.*)* were 1.5 mg/L 2,4-D, 1.5 mg/L IBA, 1.5 mg/L zeatin with 1.5 mg/L 2,4-D, and 1.5 mg/L BAP with 1.5 mg/L 2,4-D. The calli grown in those media were used for further experiments.

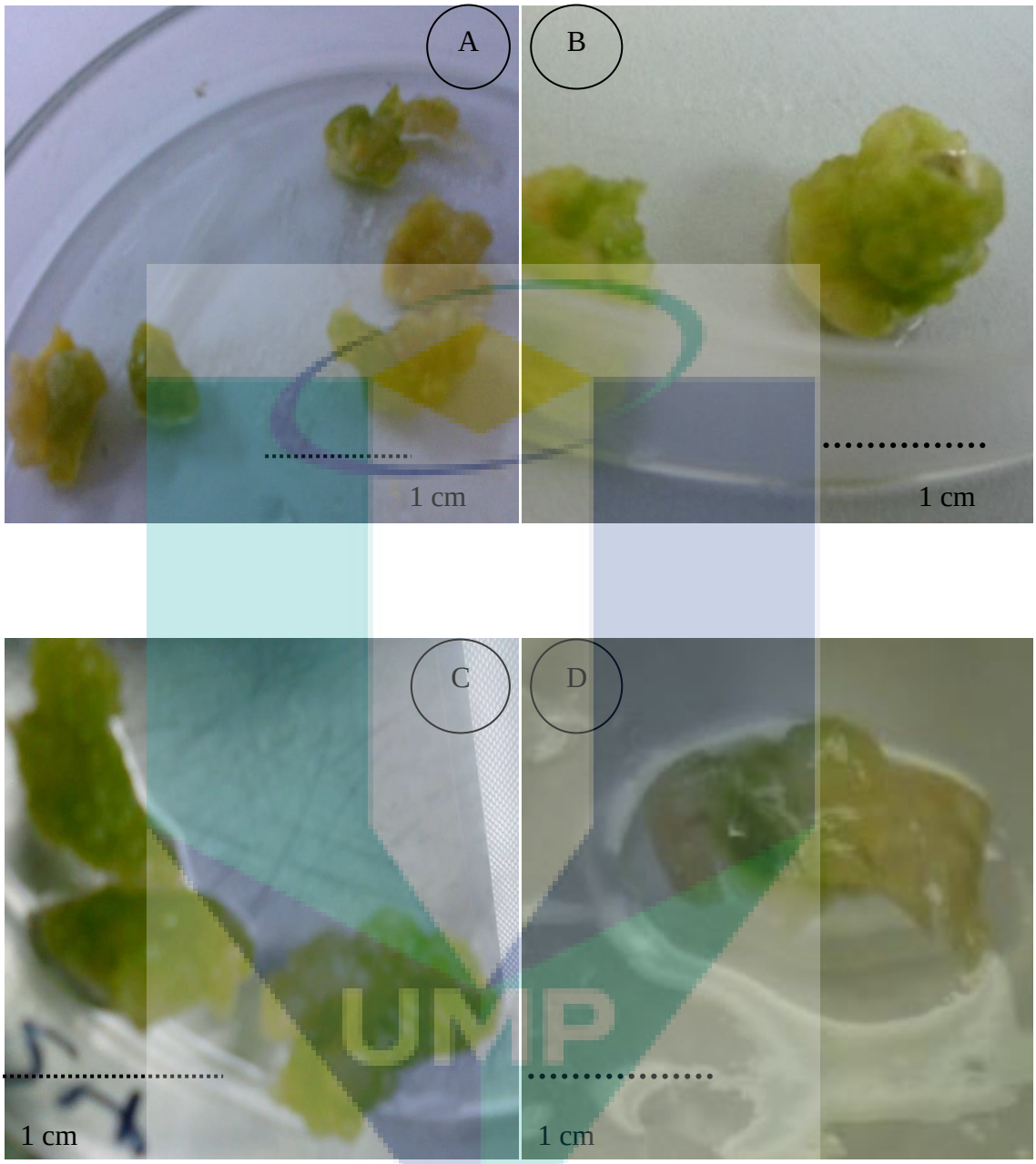


Figure 4.1: Callus induction of leaf from *Cucumis melo* L. using MS media supplemented PGRs. A) Explant in MS media without PGRs, B) Explant in MS media supplemented with 1.5 mg/L Zeatin, C) Explant in MS media supplemented with 1.5 mg/L BAP, D) Explant in MS media supplemented with 0.5 mg/L auxin (IBA). The scale (1cm) representing the explant above)

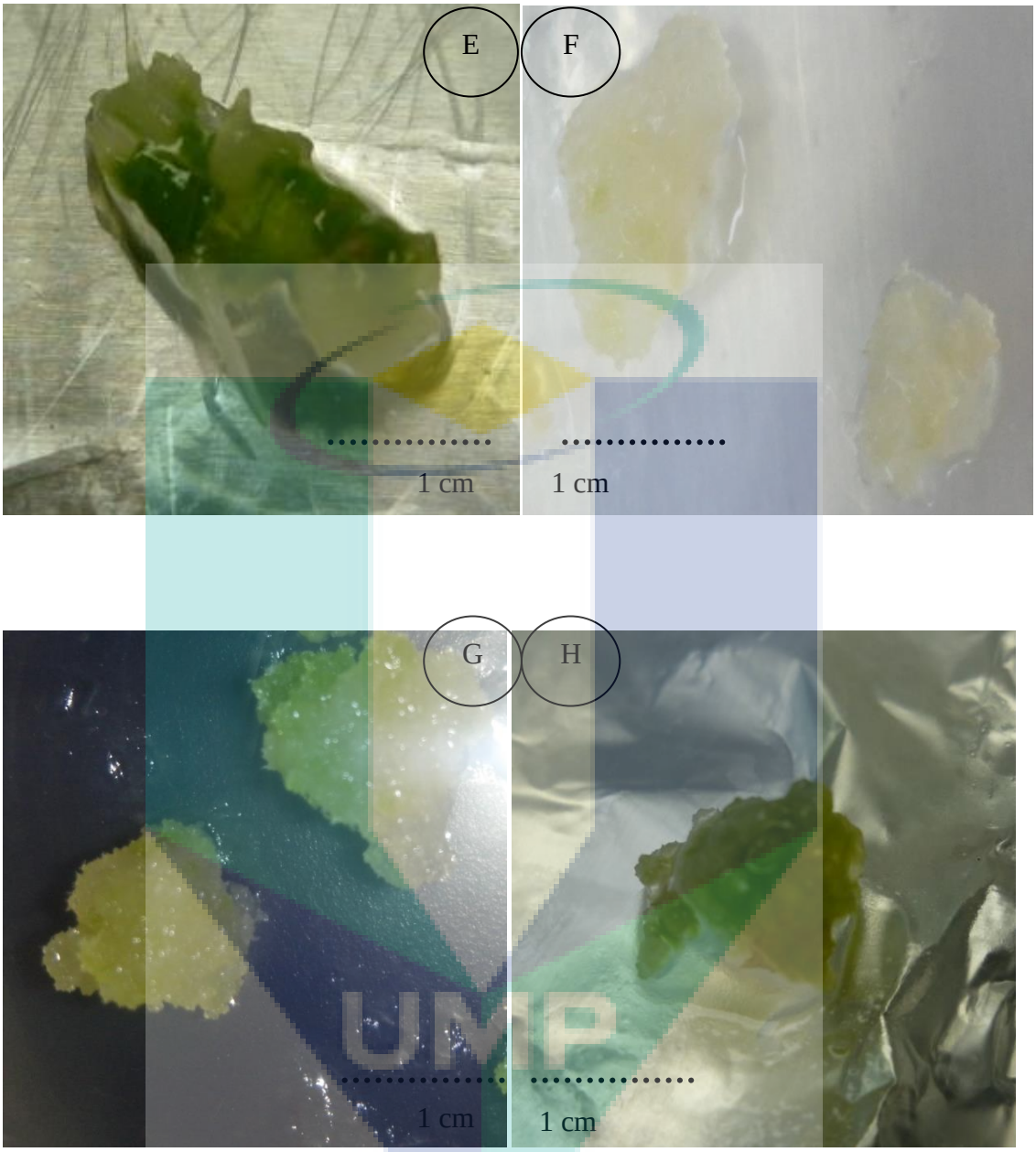


Figure 4.2: Callus induction of leaf from *Cucumis melo* L. using MS media supplemented with PGRs. E) Explant in MS media supplemented with 1.0 mg/L auxin (IBA), F) Explant in MS media supplemented with 1.5 mg/L auxin (IBA), G) Explant in MS media supplemented with 0.5 mg/L auxin (2,4-D), H) Explant in MS media supplemented with 1.0 mg/L auxin (2,4-D). The scale (1cm) representing the explant above.

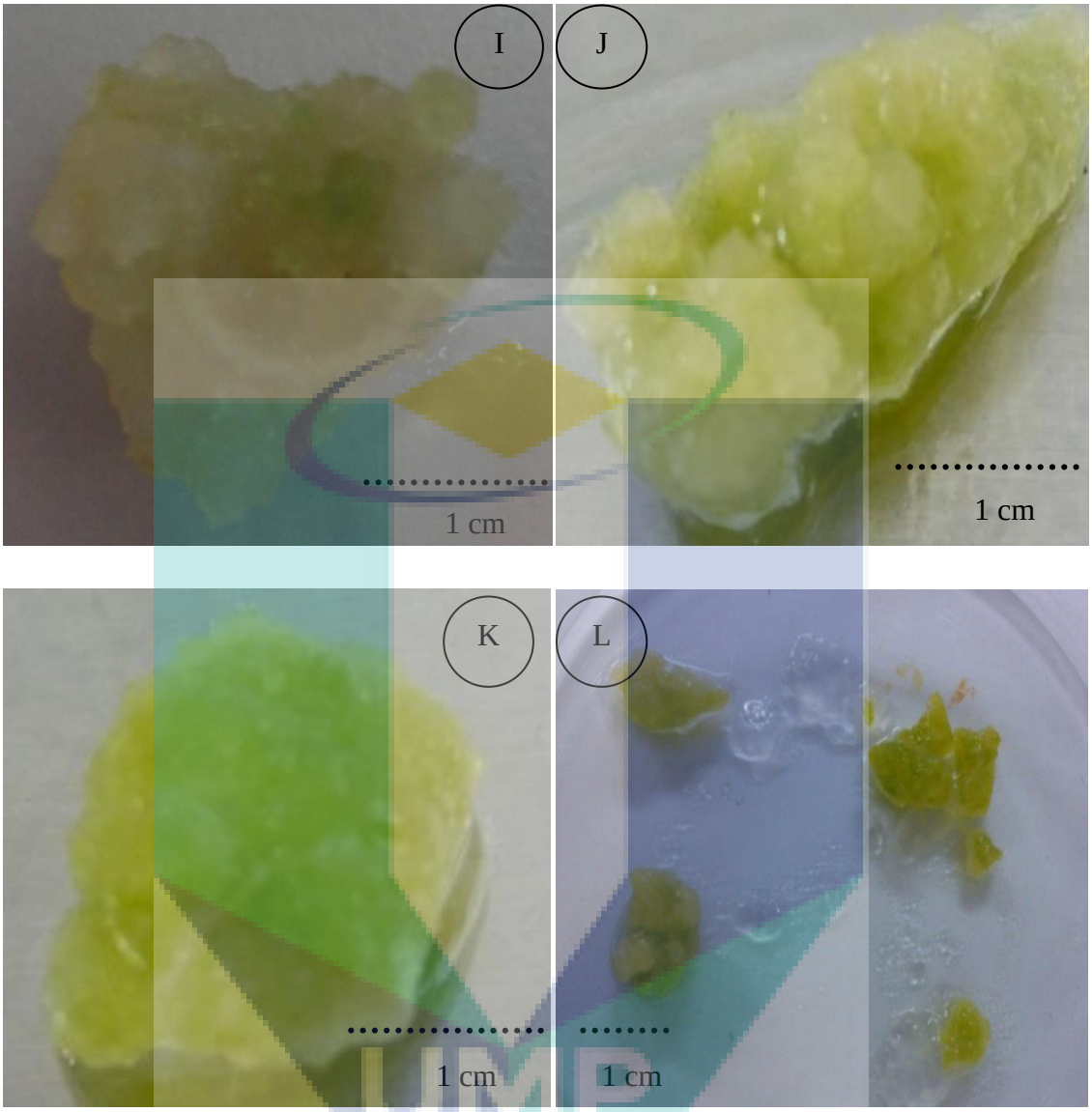


Figure 4.3: Callus induction of leaf from *Cucumis melo* L. using MS media supplemented with PGRs. I) Explant in MS media supplemented with 1.5 mg/L auxin (2,4-D), J) Explant in MS media supplemented with 1.5 mg/L Zeatin and 1.5 mg/L 2,4-D, K) Explant in MS media supplemented with 1.5 mg/L BAP and 1.5 mg/L 2,4-D, L) Explant in Media supplemented with 1.5 mg/L IBA and 1.5 mg/L 2,4-D. The scale (1cm) representing the explant above)

4.3 EFFECT OF PLANT GROWTH REGULATORS (PGRs) ON TOTAL PHENOLICS AND TOTAL FLAVONOIDS CONTENT OF THE CALLUS OF *Cucumis melo L.*

The selected concentrations of plant growth regulators (PGRs) were 1.5 mg/L 2,4-D, labelled as C4, 1.5 mg/L IBA, labelled as C3, 1.5 mg/L zeatin with 1.5 mg/L 2,4-D, labelled as C1. 1.5 mg/L BAP with 1.5 mg/L 2,4-D labelled as C2.

In this study, the secondary metabolites of callus extracts; C1, C2, C3 and C4 were examined from its extracts. Figure 4.4 and 4.5 showed callus maintenance from the first week to the fifth week. Each week, 1 g callus (C1, C2, C3, C4) were extracted with 70 % methanol and the value of total phenolics and total flavonoids content were determined. According to figure 4.4, the total phenolics content for the first week until third week of callus showed rapid increase. In figure 4.5, most of the total flavonoids content value shows gradual increase from first week until fourth week only, on fifth week, it started to decrease.

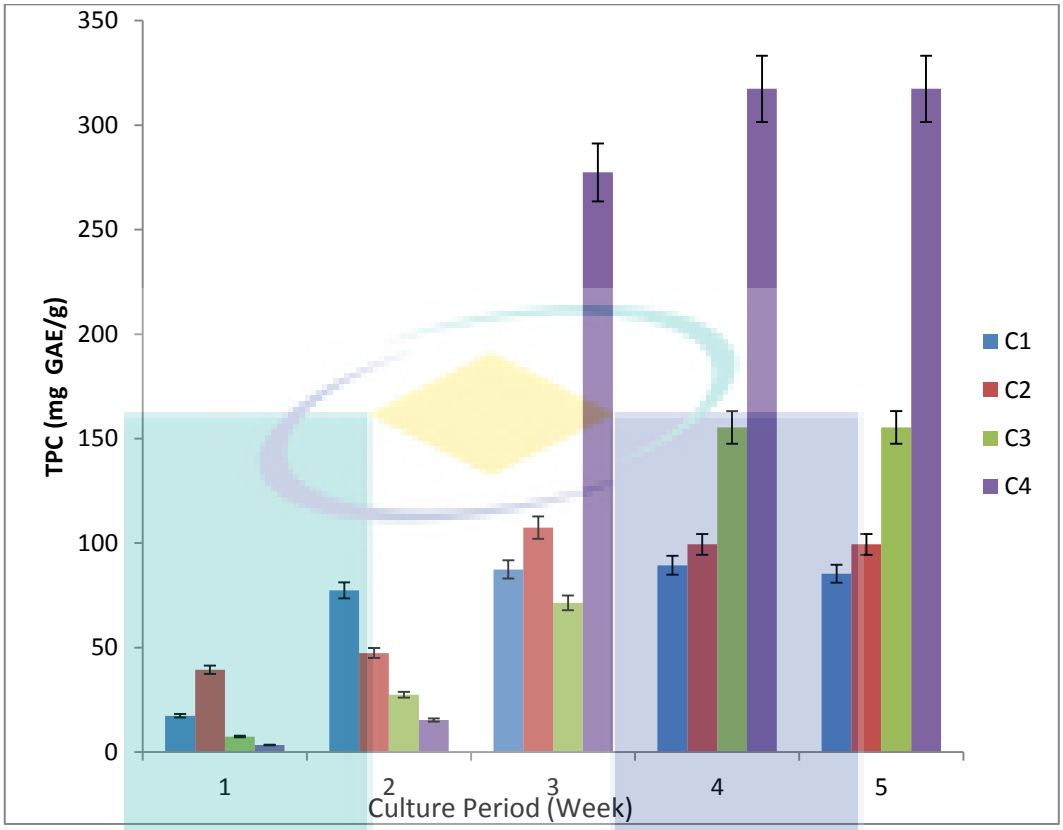


Figure 4.4 : Total phenolics content of *Cucumis melo* L. callus extracts (C1, C2, C3 and C4) cultured in MS media supplemented with PGRs incubated at pH 5.7±1, 25 ± 2 ° C with a photoperiod of 16 hours fluorescent light. The data presented were observed weekly for five weeks of cultures. Analysis of variance (ANOVA) was applied to transform data and mean separation was performed by Tukey test ($p < 0.05$) version 16.

Interestingly, in figure 4.4 ,the C2 showed the highest accumulation of total phenolics content, 39.4 mg GAE/g and C4 showed the lowest, 3.4 mg GAE/g at the first week of measurement. However, after cultured for four weeks, C4 showed the highest total phenolics accumulation, 317.4 mg GAE/g followed by C3 (155.4 mg GAE/g), C2 (99.4 mg GAE/g) and C1 (89.4 mg GAE/g). C4 showed the highest total phenolics content because the callus was supplemented with the strong auxin which producing healthy callus. C3 was supplemented with IBA which is an auxin had showed the second highest value for total phenolics content. Auxin may cause early maturation for callus that caused the higher accumulation of its phenolics content. Early maturation always related with higher accumulation of secondary metabolite production

(Parsaeimehr *et al.*, 2010). In this study, it was showed that callus in supplemented with only auxin was the best plant growth regulators to produce high accumulation of total phenolics content for *Cucumis melo*.

The lowest accumulation of total phenolics content was in C1 because of the callus was supplemented with both auxin and cytokinin (2,4-D and Zeatin) that may cause the low total phenolics content. Both callus extracts, C1 and C2 have a lower total phenolics content.

Mostly, the calli showed increasing trend in total phenolics content upto the fourth week where it reached its maximum growth (stationary phase). The synthesis of secondary metabolites in plant occurred because it was part of response in plant defences (Mandana *et al*., 2011). In the study done by Khan *et al.*, (2008), it was clear that the quantity as well as quality of plant growth regulators had a significant effect on the total phenolics content of Muskmelon callus extract.

Figure 4.5 showed total flavonoids content of the callus extracts. Flavonoids are also one of the secondary metabolites in plant. At the first week, C3 showed the highest total flavonoids content, 58.14 mg CE/g followed by C2 (52.15 mg CE/g) and C1 (11.86 mg CE/g).

C1 showed the lowest accumulation at the first week, 7.86 mg CE/g . C2 showed rapid accumulation of total flavonoids content from first to fourth week which indicated that the PGRs supplmented was the best in aiding high accumulation of total flavonoids content of callus extract from *C. melo*. However, C1 ans C4 had a lower accumulation which both were nearly reached maximum accumulation from first to the fourth week.

The C1 and C4 were less quality calli that caused lower accumulation of total flavonoids content than C2 because of the different plant growth regulators that had been supplemented to the media in those calli induced . In most cases, it was noted that

the concentrations of phenolics usually higher than the concentrations of flavonoid (Farouk *et al*., 2010). However, in this study, it was found that total flavonoids content is higher than total phenolics content in C2. The highest total phenolics content was in C2 was 107.4 mg GAE/g and the highest total flavonoids content in C2 was 145 mg CE/g. A wide variation in polyphenolic contents of the extract can be caused by the different polarity of the extracting solvents.

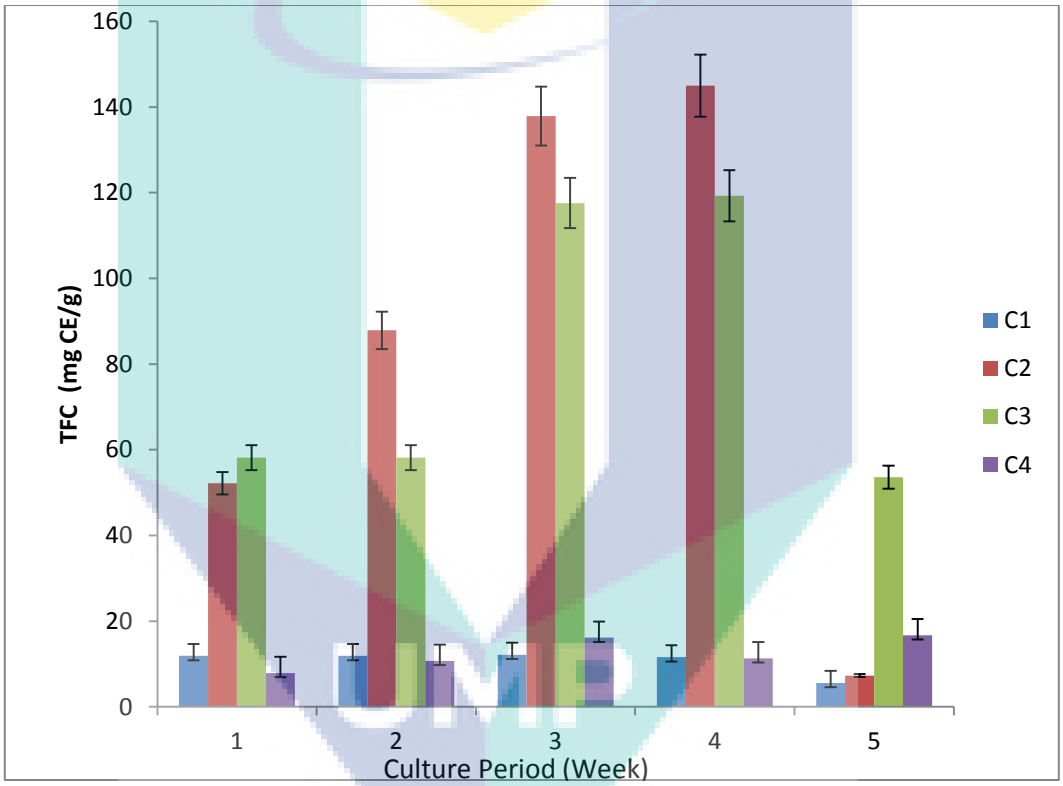


Figure 4.5: Total flavonoids content of *Cucumis melo* L. callus (C1, C2, C3 and C4) cultured in MS media supplemented with PGRs incubated at pH 5.7±1, 25 ± 2 ° C with a photoperiod of 16 hours fluorescent light. The data presented were observed weekly for five weeks of cultures. Analysis of variance (ANOVA) was applied to transform data and mean separation was performed by Tukey test ($p < 0.05$) version 16.

In previous study, a higher concentration of flavonoid was found as compared to phenolics in other plant such as Sultana *et al.* (2009), found a higher concentration of total flavonoids (1.68g/100g) in absolute methanolic extract of *Moringa oleifera* root as

compared to its total phenolics (0.22 g/100g). The variation in total flavonoid production was related with the activation of phenylalanine ammonialyase (PAL) that involved in flavonoid biosynthesis pathway (Guo *et al*., 2007). However, the activation of phenylalanine ammonialyase (PAL) in callus culture still remains unclear, whether the plant growth regulators (PGRs) create different biosynthetic pathway.

Secondary metabolite is known to be responsible for priming effect which is the ability of plant tissue culture to induce molecular mechanism of resistance to abiotic and biotic stresses, culture process or early growth of explants (Nowaki and Shulaev, 2002). In the previous study by Martin *et al.*, (2010), they indicated the ability of organism that is able to increase their adaptation to environment factors and avoid any damage. This ability determines by the regulation of hormone that important in production of lignans by *S. Chinensis* calli and biosynthesis of phenolic glycoside rhodioloside by *R. Rosea* cells (Martin et al., 2010). It suggested that phytohormone systems are major starting point for further induction of secondary metabolite production in callus culture. In this study, it was clear that at the fourth week, the calli in all extracts showed high value of total phenolics and total flavonoids content because it reach its optimal growth condition at the fourth week.

4.4 EFFECT OF PLANT GROWTH REGULATORS (PGRs) ON CALLUS GROWTH AND PHENOLIC CONTENT OF *Cucumis melo L.*

In the treatment of plant growth regulators, there were no significant difference in phenolics content of calli ($P<0.005$). The highest total phenolics and flavonoids content showed after 28th days of by most treated media . In single treatment, C3 and C4 showed the highest accumulation of total phenolics. Figure 4.6 showed mean fresh weight of of the calli. C4 showed highest mean fresh weight at fifth week, 0.19 g, where C3 was 0.09 g. C4 showed gradual increasing growth pattern from first week to fifth week where C3 showed gradual increase from first to third week, with a rapid increase from third week to fourth week and decrease at day fifth week. The fresh weights were not measured further for calli due to high contamination.

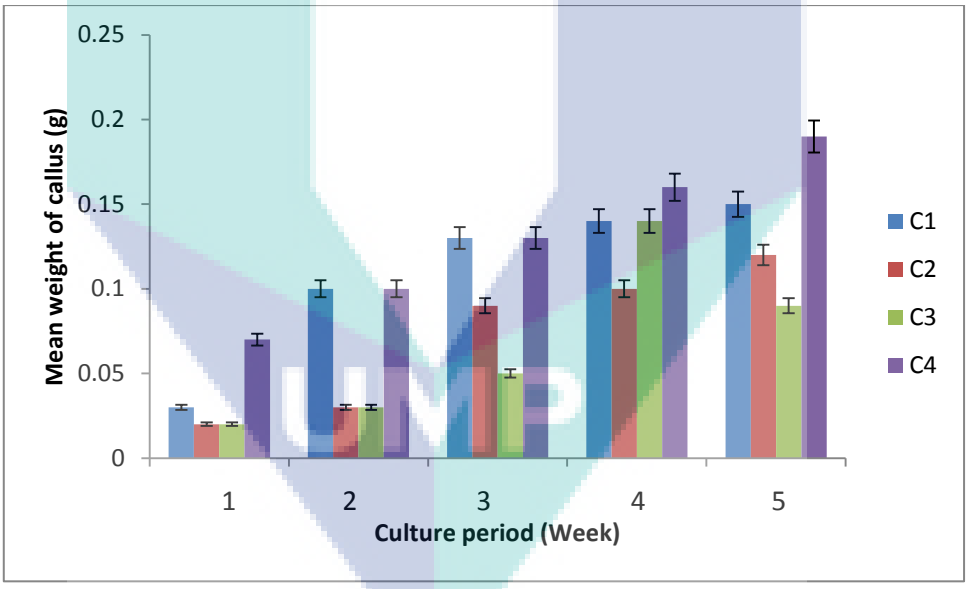


Figure 4.6: Growth of *Cucumis melo* L. callus (fresh weight) (C1, C2, C3, C4) cultured in MS media supplemented with PGRs incubated at pH 5.7±1, 25 ± 2 ° C with a photoperiod of 16 hours fluorescent light. The data presented were observed weekly for five weeks of cultures. Bar indicates the standard deviation of biomass growth from ten replicates.

When comparing total phenolics content C4 with its fresh weight, the gradual increasing pattern were quite the same except that at final week, the total phenolics content was decreased. There showed no correlation between growth of callus and its total phenolics content of C4 because at the final week of observation, the weight is increasing but the total phenolics content was decreased at the final week. This may be due to the loss of secondary metabolite of the callus as they aged (Ozyigit, 2008). It showed that the calli had reached it maximum production of phenolics at fourth week (Figure 4.4) but maximum weight at fifth week (Figure 4.6). At the fifth week, the weight increased but the phenolic accumulation was lower which indicated that the production of its secondary metabolites were slowing down as the callus aged.

When comparing the graph of total phenolics content of C3 (Figure 4.4) with its callus mean weight, the pattern were quite the same except at the final week. There showed no correlation between growth of callus and its total phenolics content because at the final week of observation, the weight was decreased and the total phenolics content was not changed. However, the result showed that the calli reached it optimum production of the phenolics at week four (Figure 4.4) as well as maximum weight at fourth week (Figure 4.6). At the final week, the weight decreased could be due to limited supply of nutrient in solid media as the callus aged. The same growth pattern was shown in the study of callus growth of *Barringtonia racemosa* by Behbahani *et al.*, (2011).

C1 callus gave the same result as C4. Figure 4.6 showed fresh weight (g) of callus in MS media supplemented with 1.5 mg/L Zeatin + 1.5 mg/L 2,4-D which were a combination of auxin and cytokinin. When comparing total phenolics content of C1 and its callus weight, the gradual increasing pattern were quite the same except that at final week. There showed no correlation between growth of callus and its total phenolics content by C1 because at the final week of observation, the weight is increasing but the total phenolics content was decreased at the final week. It showed that the calli had

reached it maximum production of phenolics at fourth week but maximum weight at fifth week.

C2 callus was cultured in MS medium supplemented with 1.5 mg/L BAP and 1.5 mg/L 2,4-D which were a combination of auxin and cytokinin. When total phenolics content of C2 with its mean weight was compared, the gradual increasing pattern shown were quite the same except that at final week, the total phenolics content was decreased. There showed no correlation between growth of callus and its total phenolics content by C2 because at the final week of observation, the weight is increasing but the total phenolics content was decreased at the final week. It showed that the calli had reached its maximum production of phenolics at fourth week (Figure 4.4) but maximum weight at fifth week (Figure 4.6).

4.5 TOTAL PHENOLICS AND TOTAL FLAVONOIDS CONTENT OF CALLUS EXTRACTS

4.5.1 Total phenolics content

One of the most important chemical compounds of plant is the phenolics which their free radical scavenging activity is due to it attributed to the hydroxyl groups. Total phenolics content of the callus extract were derived from expression (1) and expressed as g gallic acid equivalent (GAE) in fresh weight of callus extract (R^2 = 0.980). The total phenolics content of the callus extract were taken at the fourth week of callus growth because it showed the highest value among 1 to 5 week where it indicated that the callus have reach the maximum production at the fourth week.

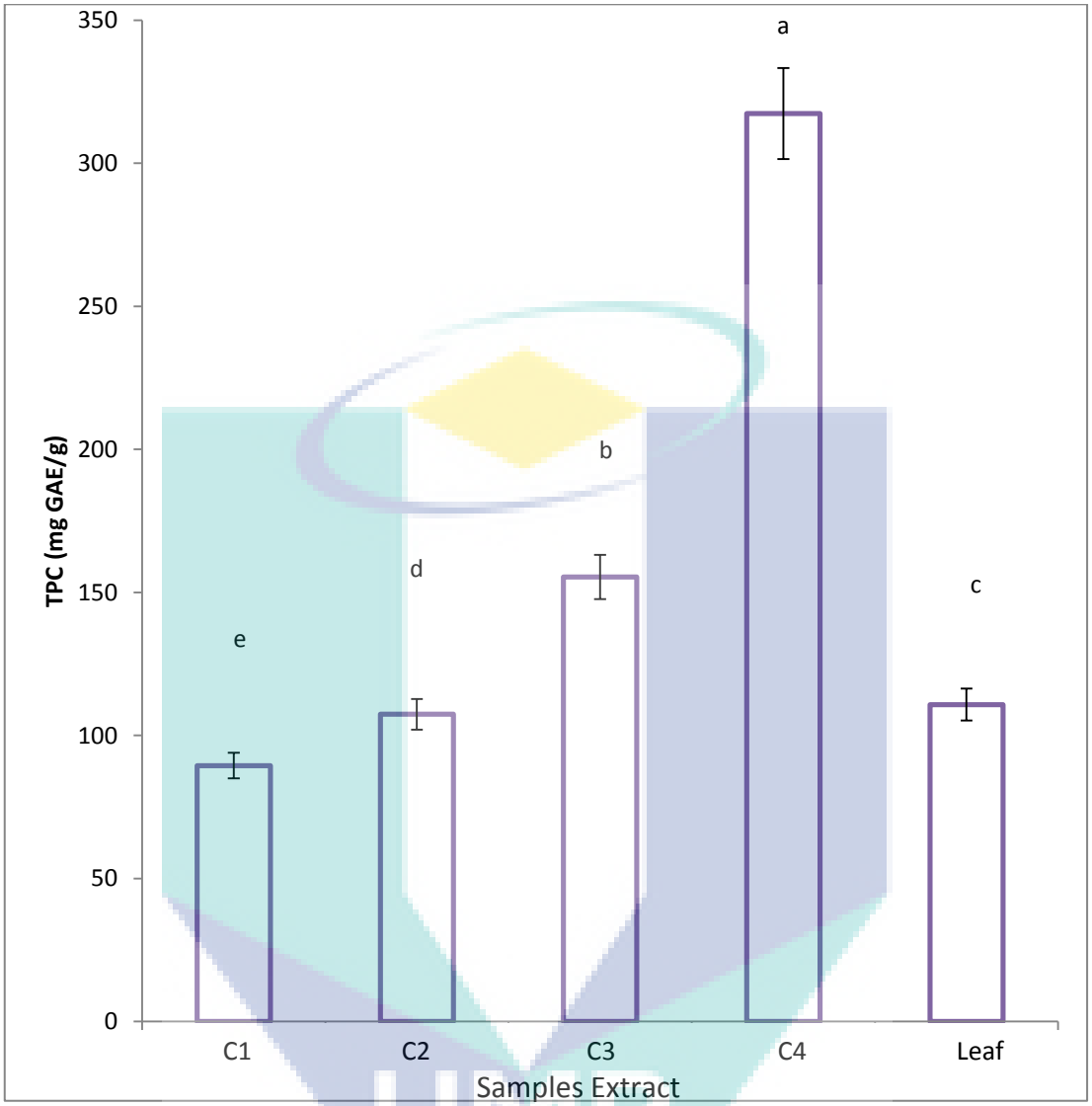


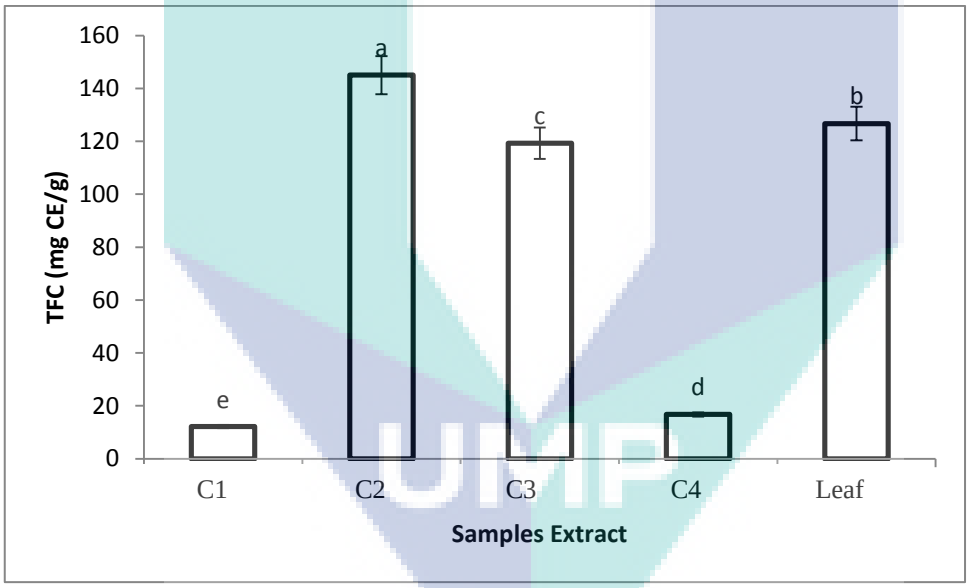
Figure 4.7: Total phenolics content of callus extracts (C1,C2,C3,C4) and leaf of *Cucumis melo* L. in different MS media (Leaf was without media). The data presented were after 28 days of cultures (except Leaf). Bar indicates the standard deviations of biomass growth are from three replicates. a>b>c>d>e, same alphabets denote no difference.

According to figure 4.7, it is shown that C4 callus extract have the highest total phenolics content, 317.4 mg GAE/g. The second highest of total phenolics content is C3 (155.4 mg GAE/g), followed by leaf (110.8 mg GAE/g), C2 (107.4 mg GAE/g) and C1

(89.48 mg GAE/g). This result showed a significantly different of total phenolics content among each callus extracts

4.5.2 Total flavonoids content

Total flavonoids content of the extracts were measured using the aluminium chloride complex formation assay by Bonhevi and Coll (1994) and Popova *et al*., (2003) with slight modification. The natural subset of phenolics is the flavonoid and it is reported that naturally, plant extract have high flavonoid content. Total flavonoids content (TFC) was derived from the expression (2) and expressed as mg catechin equivalents (CE)/g using weight of callus extract (R^2 = 0.999).



*P<0.005, statistically significant when compared between the extract

Figure 4.8: Total flavonoids content of callus extracts and leaf of *Cucumis melo* L. in different MS media (Leaf was without media). The data presented were after 28 days of cultures (except Leaf). Bar indicates the standard deviations of biomass growth are from three replicates. a>b>c>d>e, same alphabets denote no difference.

C2 callus extract has the highest total flavonoid content, 145 mg CE/g (Figure 4.8). As for total flavonoids content, the second highest is leaf (126.7 mg CE/g, followed by C3 (119.29 mg CE/g), C4 (16.71 mg CE/g), and the lowest is C1 (12.14 mg CE/g). This result showed a significantly different of total phenolics content among C1,C2,C3 callus extracts and leaf or C2, C3, C4 callus extracts and leaf. Flavonoid is the most abundance polyphenols in plant and responsible for various roles in plant such as colour, aroma of flowers as well as fruit for dispersion; help in seed germination, spore germination, growth and development of seedling. There are various factors that affect different content of polyphenols in plant. It is reported that, flavonoid is the most abundance compound in plant extract (Cakir *et al.*, 2003).

4.6 ANTIBACTERIAL ACTIVITY

Disk diffusion assay display the antibacterial activity through the inhibition zone. The biggest inhibition zone indicated that the antibacterial agent is the strongest against the bacteria tested. According to table 4.3, C4 shows the strongest antibacterial activity among all the callus extract, where it was effective against *Enterococcus faecalis* and *Salmonella typhi*. C1 and C2 were the most ineffective towards the bacteria. The biggest inhibition zone was gained by C4 against both *Enterococcus faecalis* and *Salmonella typhi* was 30 mm at highest concentration (50 mg/ml). C3 was effective against *Bacillus subtilis* where at concentration of 12.5 mg/ml the inhibition zone was 15 mm. Figure 4.9 showed inhibiton zone by C3 and C4.

In the antimicrobial study done using seed extracts of *Cucumis melo*, the extracts showed potent antimicrobial studies against *P. aeroginosa* and moderate activity against *E. coli* at the concentration of 25 mg/ml and 50 mg/ml respectively (Sabrin, 2009). However, in this study, there were no inhibition zone shown for antibacteria test against *P. aeroginosa* and *E.coli*. Both bacteria are from Gram Negative bacteria which were known to be more resistant to antibacterial agent compared with the Gram Positive bacteria.

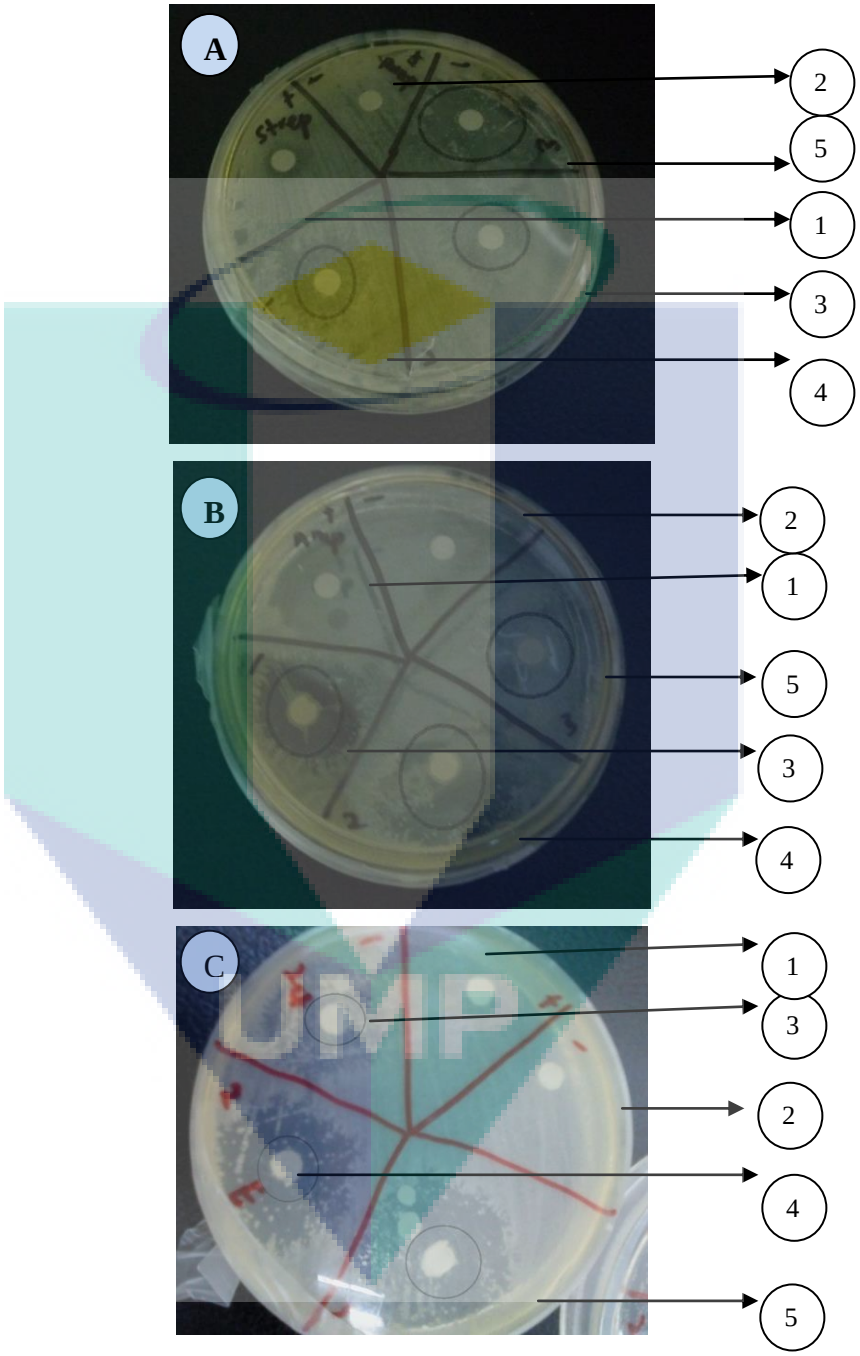


Figure 4.9: Zone of inhibiton. A: C4 with *S.typhii* B: C4 with *E. faecalis* , C: C3 with *B. subtilis.* 1: Positive control, 2: Negative control, 3: Inhibition zone for extract concentration at 12.5 mg/ml, 4: Inhibition zone for extract concentration at 25 mg/ml, 5: Inhibition zone for extract concentration at 50 mg/ml

Table 4.3: Zone of inhibition by callus and leaf extracts on different types of bacteria.

Extract	Zone of inhibition (mm)																	
	Gram positive *S. areus* ATCC baa1026			*E .faecalis* ATCC 14506			*B. subtilis* ATCC 11774			Gram negative *E. coli* ATCC 10536			*S. typhi* ATCC 1331			*P. aeroginosa* ATCC 15442		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
C1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3	-	-	-	-	-	-	15 ± 0.6	22±1	23 ± 1.5	-	-	-	-	-	-	-	-	-
C4	-	-	-	18±0.6	25±0	30 ± 1	-	-	-	-	-	-	20±1.7	25±1	30±0.6	-	-	-
Leaf	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SM (1mg/ml)	22±1.5	-	-	35 ± 1	-	-	24± 0.6	-	-	31±1	-	-	16±0.6	-	-	22±0	-	-
-ve	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Values are mean ± standard deviation of triplicates (n= 3)
1: 12.5mg/ml of extract
2:25mg/ml of extract
3:50mg/ml of extract
SM : Streptomycin (positive control)
-ve: Negative control
- : No Inhibition

Minimal inhibitory concentration (MIC) is a test for antibacterial activity to determine the lowest concentration of the extract that is able to inhibit the bacterial growth. *S. aureus* is known as a pathogen for human skin. MIC value is taken from the lowest concentration of the extracts that can inhibit the growth of the bacteria by using micro plate reader. The turbidity showed in the extracts solution with the bacteria indicated that the extracts were ineffective to inhibit the growth of bacteria. Table 4.4 showed the result of minimal inhibitory concentration. The lowest concentration of callus extract that could inhibit bacterial growth was determined. Different callus extracts have different minimal concentration value that could inhibit the growth of bacteria.

Table 4.4: Minimal Inhibitory Concentration on different types of bacteria

Extract	Minimal inhibitory concentration (MIC) (mg/ml)					
	Gram positive			Gram negative		
	S. areus	*E .faecalis*	*B. subtilis*	*E. coli*	*S. Typhi*	*P. aeroginosa*
	ATCC baa1026	ATCC 14506	ATCC 11774	ATCC 10536	ATCC 1331	ATCC 15442
C1 (mg/ml)	-	-	-	-	-	-
C2 (mg/ml)	-	-	-	-	-	-
C3 (mg/ml)	-	-	12.5±0.5	-	-	-
C4 (mg/ml)	-	12.5±0.5	-	-	25±1	-
Leaf (mg/ml)	-	-	-	-	-	-
-ve (mg/ml)	-	-	-	-	-	-
SM (mg/ml)	1	1	1	1	1	1

Values are mean ± standard deviation of triplicates (n = 3)
-: No inhibition

According to table 4.4, in the antibacterial test of the extracts with *S. aureus*, all of the extracts showed no inhibition towards the bacteria growth. It is the same with the study against *P. aeroginosa* and *E. coli*. Interestingly, in the study of the extracts against *B. subtilis*, C3 shows antibacterial activity which gave minimal inhibitory concentration's value of 12.5 mg/ml. While, in antibacterial test against *S. typhi* and *E. faecalis*, C4 gave the minimal inhibitory values which were 25 mg/ml and 12.5 mg/ml. C4 shows lowest minimal inhibitory concentration value against two types of bacteria tested.

It were founded that the Gram Negative bacteria were the most resistant towards the callus extract. It is reported that the Gram Negative bacteria are more resistant towards the antibacterial agent due to its double membrane surrounding each bacterial cell that excludes certain drugs and antibiotics from penetrating the cell (Melissa, 2000). *E. coli* has been known to be multiresistant toward antibacterial agent (Gislene *et al*.,2000), was also resistant toward the callus extract. All the callus extracts gave no inhibition effects towards *E.coli* and *P.aeroginosa*. So, it was suggested that both bacterial strains may have resistant mechanism towards the extracts and the concentrations of the extracts used may be lesser to inhibit the bacterial activity.

C4 shows the strongest antibacterial activity towards the bacteria. It showed that it have different mechanism to inhibit the growth of the bacteria. It could be related to its total phenolics or total flavonoids content. Hence, C4 extract contains highest total phenolics content compared with the other extracts where C1 and C2 had the lowest total phenolics content. Phenolic acid is known for many bioactivities which is included antibacterial activity (Kanti and Syed., 2009).

In both experiment of disk diffusion and minimal inhibitory concentration, the leaf extract of the *C. melo* did not show any inhibition compared with the callus extracts which suggested the callus extracts were stronger antibacterial agent than the leaf extract of the plant. There are no study supported the use of *C.melo* leaf as antibacterial

agent has been publish to date except the plant from the same genus (Kumar and Kamaraj, 2011).

4.7 ANTIHYPERTENSIVE ACTIVITY

Antihypertensive activity was determined by calculating the percentage of inhibition of the angiotensin converting enzyme (ACE). Figure 4.10 showed the percentage of inhibition rate of callus extracts and leaf extract of *Cucumis melo* at 50 mg/ml to angiotensin converting enzyme (ACE). Samples were considered antihypertensive if ACE inhibition was 50 % or higher by the extracts (Braga *et al.*, 2007). All the five samples gave inhibition value ranges from 50 to 70 %. C4 showed the highest inhibition rate among the four types of callus extracts which is 70.32 %.. Followed by C1 and C2 extracts inhibition rate are 63.45 % and 56.09 % respectively. Meanwhile, the C3 callus extract showed the lowest inhibition rate which was only 50.78 % so it have the highest ACE converting rate, 49.22 %

The ACE inhibition of leaf extract was shown in Figure 4.10, where the inhibition rate was 69.43 %. The C4 callus extract showed higher inhibition rate compared to leaf extract of the plant. This suggested that the C4 callus extract exhibited stronger ACE inhibitor than the leaf extract of the plant. There were no study stated the popular use of *Cucumis melo* as antihypertensive has been publish until to date. C4 also contain higher total phenolics content than the leaf extract and other callus extracts. It was found that cocoa plant had antihypertensive effect because of its high polyphenols content (Cienfugus *et al*., 2009). This suggests that high phenolics content in the extracts might contribute to high inhibition effect towards ACE.

The flavan-3-ols and procyanidins compound existed in plant extracts are related to antihypertensive activity (Goretta *et al*., 2003). Compound that also usually involved in antihypertensive property are 2, 4-Dihydroxy-2,5-dimethyl-3(H)-furan-3-

one and acetic acid hydroxyl-methyl ester. Both compounds are reported to have antihypertensive activities which are able to inhibit ACE (Kumar *et al*., 2010).

Captopril was used as the standard in this experiment. The absorbance measurement was use in the substrate-enzyme-inhibitor mixture. Angiotensin converting enzyme (ACE) inhibitor works to inhibit Angiotensin I from becoming Angiotensin II which caused hypertension. The higher inhibition rate showed by the extract indicated that it had higher antihypertensive activity.

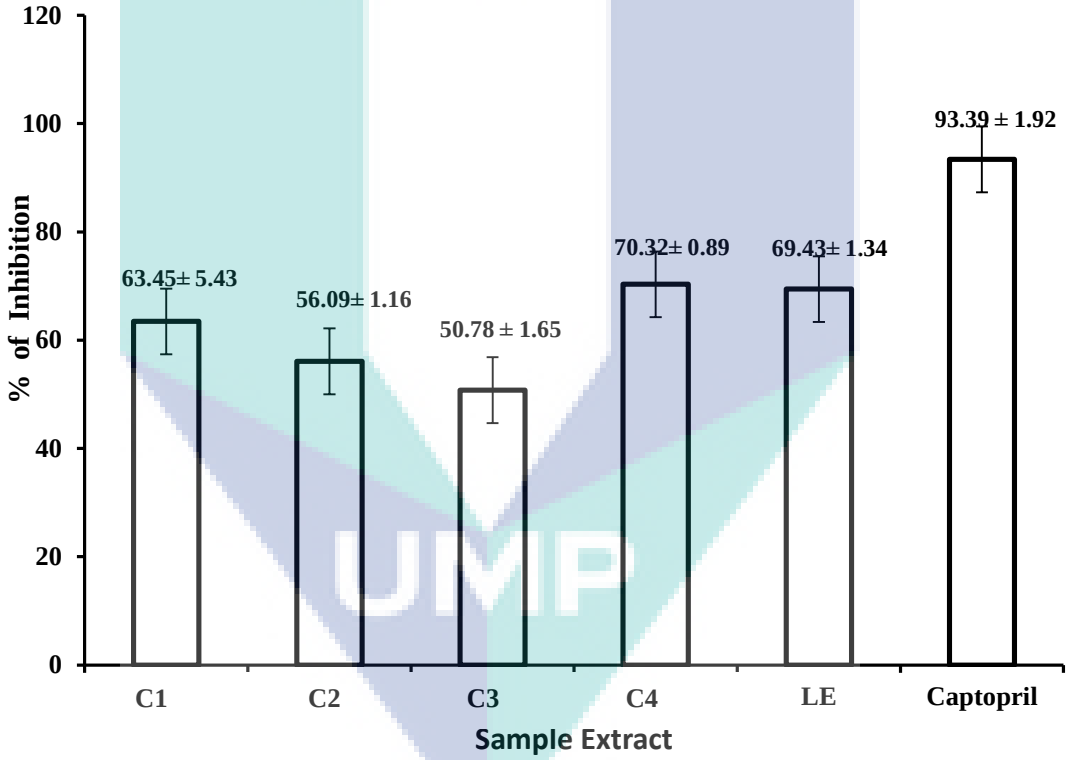


Figure 4.10: Percentage (%) of inhibition of ACE by callus extracts and leaf extracts of *Cucumis melo* L. C1, C2, C3,C4 and **LE** Leaf extract, and Captopril at concentration of 50 mg/ml. Bar indicated the standard deviations of inhibition rate were from three replicates.

According to the figure 4.8, all of the extracts were able to inhibit the ACE because the percentage of inhibition was more than 50 %. The results suggested that the

extracts may contain compounds that are able to inhibit the ACE. It also indicated that some of the compound for antihypertensive may exist in the extracts that enable in inhibiting the ACE.

The inhibition of ACE by C2 was higher than C3. However, the total phenolics and total flavonoids of C3 were higher than C2. This was maybe due to the existence of more phenolics acid compound that possessed antihypertensive activity in C2 were more than C3. It was applied to the result of C1 where the inhibition was higher than C3 and C2. C1 had the second highest total flavonoids content and the lower phenolics content than C2 and C3 . It was reported that there are certain phenolics acids that possessed strong bioactivities (Syed and Kanti, 2009). Some of the compounds that possessed strong antihypertensive activity might be discovered which exist in the extracts.

4. 8 CHEMICAL PROFILING

A HPLC connected to a UV-vis DAD was employed to profile and quantify gallic acid (phenolics acid) and catechin hydrate (flavonoid) in the callus and leaf methanolic extracts of *C. melo*. Liquid chromatography was used in this study in order to examine the profile, identification and quantification of the bioactive compounds. The commercial standards, gallic acid (GA) and catechin hydrate (CE) were used in quantification. In order to determine the concentrations, the peak areas were calculated which were proportional to the amount of analyze in a peak.

Figure 4.11 showed authentic chromatogram of standard gallic acid, and figure 4.12 showed catechin hydrate. The retention time for gallic acid (GA) was at 5.278 minute. The retention time for catechin hydrate was at 7.836 minutes.

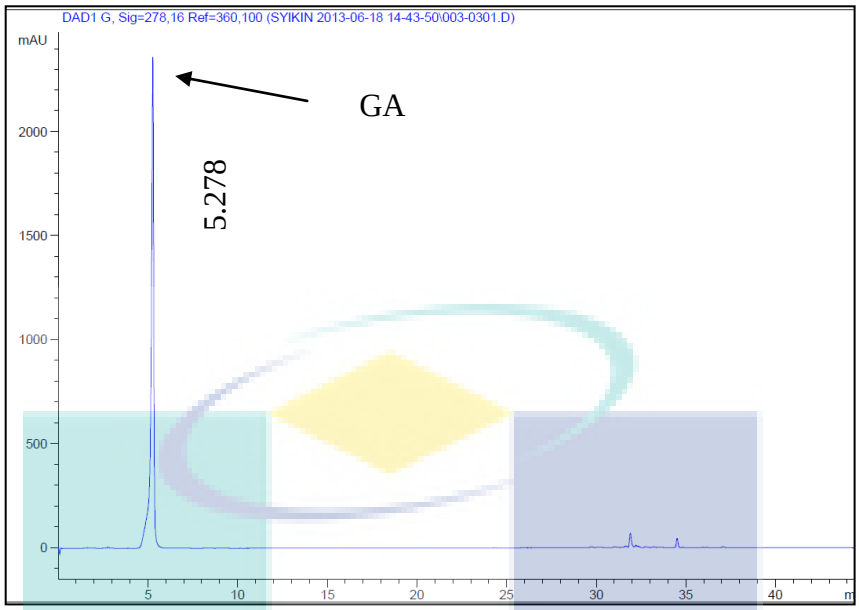


Figure 4.11: HPLC chromatogram containing Gallic acid (200 µg/ml). Retention time (5.278 min) (λ= 278nm)

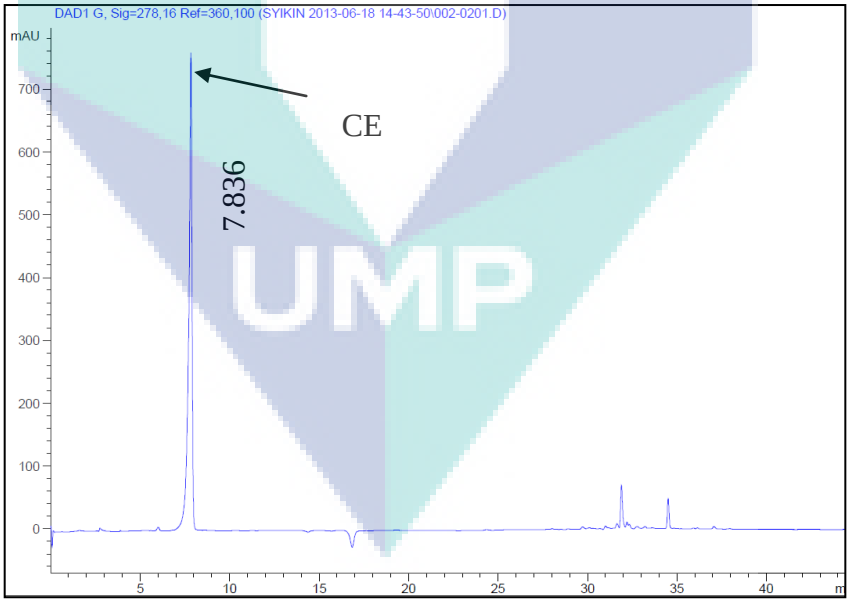


Figure 4.12: HPLC chromatogram containing Catechin hydrate (200 µg/ml). Retention time (7.836) (λ= 278nm)

The results of HPLC analysis for the calli showed that the highest concentration of gallic acid was found in callus extracts of C4 (3.68 µg/ml) followed by C1 (3.57 µg/ml), C2 (3.42 µg/ml) and C3 (3.33 µg/ml) (Figure 4.13). Meanwhile, the results of HPLC analysis for the calli showed that the highest concentration of catechin hydrate was found in callus culture in C2 (39.55 µg/ml) followed by C3 (39.50 µg/ml) and C4 (39.45 µg/ml) (Figure 4.13). Catechin hydrate was not detected in C1 and the leaf extract may be due to too low concentration of the compound in callus and the leaf extract. High concentration of catechin and gallic acid were highly correlated with callogenesis in callus culture of *Gossypium hirsutum* L. (Tanoh *et al*., 2009). Polyphenols concentrations were also found higher in callus culture of *Crataegus mogyna* (Hawthorn) than its plant parts (Bahorun et al., 2003). This was because the callus culture was grown *in vitro* with culture conditions involved controlling chemical and physical factors that resulted in production of high polyphenols compared with those produced by intact plant (Tsalakostal, 2009).

As a conclusion, the callus extracts of C4 contained the highest concentration of gallic acid compared with other callus extracts. The callus extract of C2 contained the highest concentration of catechin hydrate compared with other callus culture and the leaf extract of the plant.

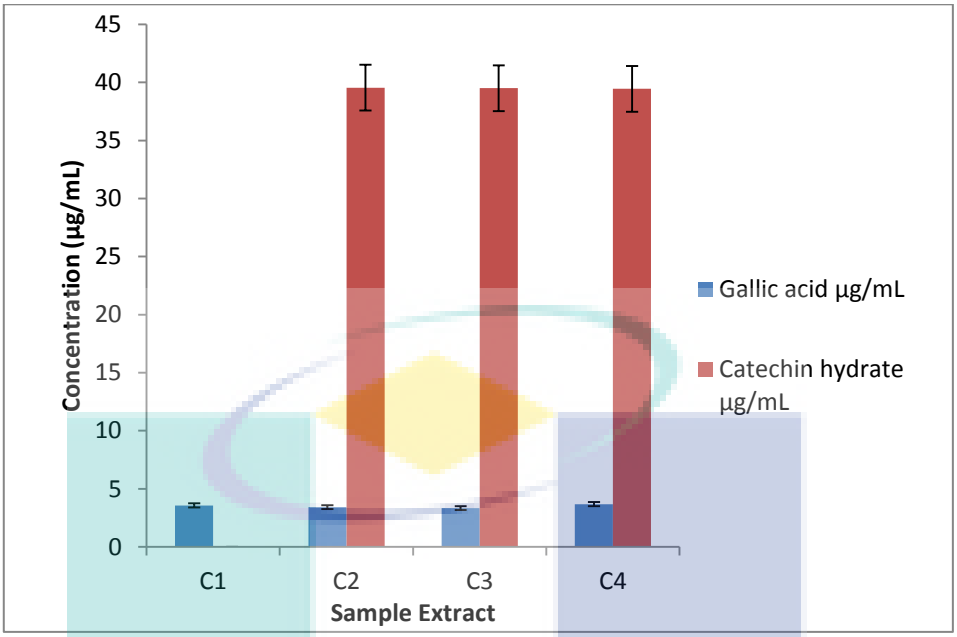


Figure 4.13: Gallic acid and Catechin hydrate concentration in callus extracts of *Cucumis melo*. Bar indicated the standard deviation of gallic acid concentration from three replicates

4.9 RELATIONSHIP OF ANTIBACTERIAL AND ANTIHYPERTENSIVE ACTIVITIES WITH TOTAL PHENOLICS AND TOTAL FLAVONOIDS CONTENT

There are a number of phytochemicals compounds that may presented in the extracts that have the antimicrobial and angiotensin converting enzyme (ACE) inhibitors reported in the previous study (Kanti and Syed, 2009). The callus extract may contain many secondary metabolites that may contribute to various strong bioactivities.

The C1 possessed highest antibacterial and antihypertensive activities compared with other extracts even with the leaf extract of the plant itself. This extracts had high total phenolics and low total flavonoid content. It was described by Kaur Manpreet *et al*., (2011), that high total phenolics and total flavonoids content usually related to high antioxidant activity. The high antioxidant activity relation to many bioactivities were already known, such as antimicrobial and antihypertensive. The correlation test for total

phenolic and antibacterial properties gave positive correlation, where $r^2 = 0.966$. Which mean higher total phenolic content will gave higher antibacterial properties.

Most of the callus extracts possessed both phenolics and flavonoid content in their respective concentration. It was reported that phenolics compound contributed in inhibitory of mutagenesis and carcinogenesis in human because it might contributed directly in antioxidative action (Tanaka *et al.,* 1998). It was reported the same for flavonoids compound (Cakir *et al*., 2011)

There are a number of plant compounds that showed antibacterial and antihypertensive properties. Phytol is one of the compounds that has long been studied for antimicrobial activity and linoleic acid is related with healthy food. Some of the plant compounds can work coexistence of other compounds which is also called as having synergistic effect such as benzoic acid with salicylic acid.

According to the study, it was also shown that callus induced from different plant growth regulator had different total phenolics and total flavonoid content. It is clearly shown that plant growth regulators play an important role for production of phytochemicals. It was stated that there are numerous factors that might affect polyphenols content in plant which environmental factor was one of the major effect. Environmental effects are rain fall, sunlight exposure or agronomic (culture in greenhouses or field, optimised environment, biological culture). It was reported that plants that are grown by organic or sustainable environment can produce higher polyphenols than conventional plant. (Kanti and Syed,2009). Arora *et al*., (2011), reported that methanolic seed extract of *Cucumis melo* contained high triterpenoid. There are also flavonoids, carbohydrate, proteins and phytosterol present in the methanolic seed extracts.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The results provide evidences that the callus extract of the *Cucumis melo* L has potential compounds for angiotensin converting enzyme inhibitors and antibacterial agents such as phenolics acid and flavonoid. The result also demonstrated that callus extract can inhibit bacterial growth and inhibit the ACE. Plant tissue culture medium that contain 1.5 mg/L 2,4-D was an optimum concentrations which were able to induce callus formation. The bioactivity involved by the callus extract would be contributed by its polyphenols or other phytochemicals as well as it mineral contents.

. The C4 callus extracts contains the highest value of total phenolic content but lowest total flavonoid content, highest antibacterial and antihypertensive activities The HPLC profile shows that phenolics (Gallic acid) were presented in extracts C1, C2, C3, and C4 and flavonoids (Catechin hydrate) were presented in C2, C3,C4.

As a conclusion, the methanolic extracts from callus of muskmelon have the potential market in the natural product industry for antibacterial and antihypertensive treatments.

5.2 RECOMMENDATIONS

1) One of the best ways to know the effectiveness of the extract towards bioactivity test is to extract the pure compound from the callus. This is in order to detect individual compound that is responsible to the antibacterial or antihypertensive properties. Furthermore, the compound can be further tested for its stability or effect towards plant and animal cells.

2) To test for *in vivo* antihypertensive test. The *in vivo* study for antihypertensive activity is recommended in order to learn the side effects of the callus extract towards animal cell. *In vitro* test had been done in the experiment for antihypertensive test. *In vivo* test can be done to determine the effectiveness of the compound in animal cells. The test can be done using hypertensive induced rat. The *in vivo* test has a different approach towards the way of the extract work. The result can be compared with *invitro* test.

3) To test callus extract for anticancer test. Cancer research had been done for many years using plant extracts. It is important to find the anticancer agent in order to fight against cancer. The anticancer test can be done using the callus extract to determine whether it have the anticancer properties.

4) The HPLC profiling cannot give the name of the compounds that presented in the extracts. So, the presented compound in the extract can be verified by other methods. Further characterization of phytochemicals or polyphenols presented in the callus extract can be study using liquid chromatography mass spectrometry (LCMS) as it can identify the compound in the extract or nuclear magnetic resonance spectroscopy (NMR).

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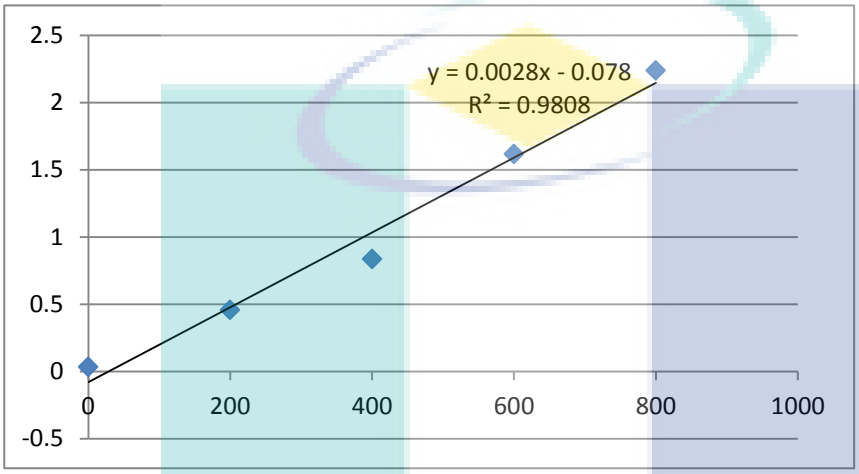
APPENDIX A

Gallic acid concentration and absorbance value

No.	Concentration (µg/ml)	Absorbance value
1	0	0.0325
2	200	0.456
3	400	0.8355
4	600	1.6141
5	800	2.235



No.	Code Sample	Weight (g)	Vol.extract (ml)	Vol.analysis (µl)	Absorbances	a	b	TPC(mg GAE/100 g)	TPC (mg GAE/g)
1	Fruit	0.02	10	1000	0.028			1340.00	13.40
2	Stem	0.02	10	1000	0			1940.00	19.40
3	Leaf	0.02	10	1000	0			10940.00	109.40
4	z	1.0	10	1000	0.065			8740.00	87.40
5	b	1.0	10	1000	0.073			10340.00	103.40
6	iba	1.0	10	1000	0.099			15540.00	155.40
7	2,4d	1.0	10	1000	0.18	0.0005	0.0213	31740.00	317.40

Gallic acid standard curve

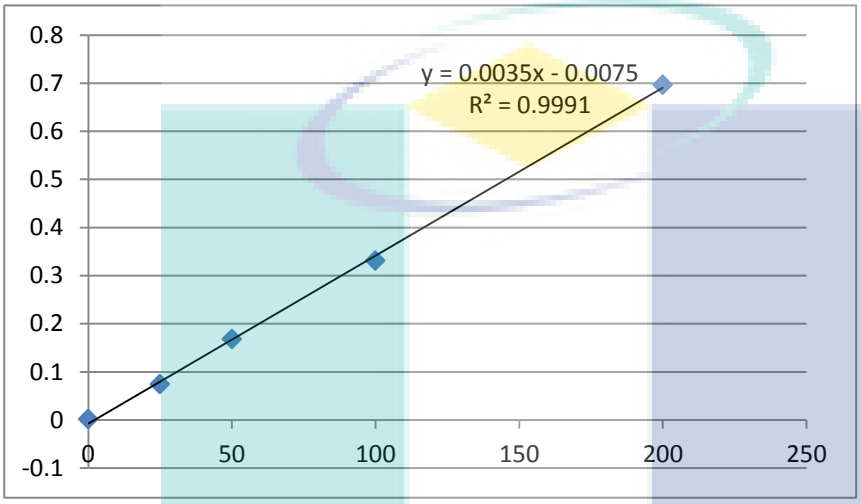
Absorbance: 0.002gallic acid (mg) -0.078 (4.9.1)

Formula: C=C1 × V/m.

Where; C=Total content of phenolic compounds in mg/g, in GAE (gallic acid equivalent); C1=The concentration of Gallic acid established from the calibration curve in mg/ml; V=The volume of extract in ml; M=The weight of plant extract in g

Catechin hydrate concentration and absorbance value

No.	Concentration (µg/ml)	Absorbance value
1	0	0.002
2	25	0.074
3	50	0.168
4	100	0.331
5	200	0.696



Catechin hydrate standard curve

No.	Code Sample	Weight (g)	Vol.extract (ml)	Vol.analysis (µl)	Absorbances	a	b	TF(mg GAE/100 g)	TF (mg GAE/g)
1	Fruit	0.02	10	1000	0.064			2042.86	20.43
2	Stem	0.02	10	1000	0.117			3557.14	35.57
3	Leaf	0.02	10	1000	0.436			12671.43	126.71
4	z	1.0	10	1000	0.35			10214.29	102.14
5	b	1.0	10	1000	0.475			13785.71	137.86
6	iba	1.0	10	1000	0.404		-	11757.14	117.57
7	2,4d	1.0	10	1000	0.049	0.0035	0.0075	1614.29	16.14

Total flavonoid content (mg catechin/g)= $\frac{\text{Catechin hydrate (mg/L) x total volume of methanol extract (mL x 10-3(L/ µl) x dilution factor}}{\text{Sample weight (g) x 10-3(mg/g)}}$

Absorbance = 0.003x catechin (mg) – 0.007 (4.9.2)

CALLUS INDUCTION

Table A1: Weight in combination of 2,4-D and Zeatin (Z)

Treatment		N	Mean weight of callus
ZEATIN	2,4-D		Days 30 (Mean + S.E)
0	0	6	0.10 ± 0.08[a]
0	0	6	0.03 ± 0.00[a]
0	0	5	0.04 ± 0.01[a]
1.5	1.5	9	0.30 ± 0.09[b]
1.5	1.5	4	0.07 ± 0.02[a]
1.5	1.5	7	0.03 ± 0.03[a]
1.5	1.5	8	0.03 ± 0.00[a]
1.5	1.5	6	0.14 ± 0.02[ab]
1.5	1.5	3	0.11 ± 0.01[a]
1.5	1.5	4	0.10 ± 0.03[a]
1.5	1.5	3	0.15 ± 0.01[ab]
1.5	1.5	5	0.19 ± 0.08[ab]
1.5	1.5	6	0.14 ± 0.01[ab]
1.5	1.5	5	0.10 ± 0.02[a]
1.5	1.5	5	0.13 ± 0.02[ab]
1.5	1.5	9	0.15 ± 0.01[a]

Result are mean ± SE of 16 treatment with 10 replicates each.
Means in a column followed by the same letter are not significantly different (P= 0.05) according to Duncan's multiple range test.

ANOVA

Weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.860	15	.191	6.751	.000
Within Groups	2.372	84	.028		
Total	5.232	99			

[DataSet1] D:\2,4D + Zeatin\SPSS\Weight\weight.sav

Descriptives

Weight

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0 (control)	6	.10417	.194995	.079606	-.10047	.30880	.007	.500
0 (control)	6	.02750	.008803	.003594	.01826	.03674	.017	.041
0 (control)	5	.03560	.011371	.005085	.02148	.04972	.021	.051
1.5 24d +1.5 Zeatin	9	.29567	.261653	.087218	.09454	.49679	.062	.882
1.5 24d +1.5 Zeatin	4	.06625	.045529	.022765	-.00620	.13870	.021	.113
1.5 24d +1.5 Zeatin	7	.02929	.008261	.003122	.02165	.03693	.018	.041
1.5 24d +1.5 Zeatin	8	.03412	.005515	.001950	.02951	.03874	.027	.042
1.5 24d +1.5 Zeatin	6	.13650	.040139	.016386	.09438	.17862	.099	.213
1.5 24d +1.5 Zeatin	3	.10667	.019757	.011407	.05759	.15575	.089	.128
1.5 24d +1.5 Zeatin	4	.09900	.063177	.031589	-.00153	.19953	.032	.173
1.5 24d +1.5 Zeatin	3	.14533	.016258	.009387	.10495	.18572	.131	.163
1.5 24d +1.5 Zeatin	5	.18580	.183739	.082171	-.04234	.41394	.012	.436
1.5 24d +1.5 Zeatin	6	.13700	.035519	.014501	.09973	.17427	.082	.189
1.5 24d +1.5 Zeatin	5	.10180	.055138	.024658	.03334	.17026	.032	.182
1.5 24d +1.5 Zeatin	5	.13000	.055637	.024882	.06092	.19908	.051	.203
1.5 24d +1.5 Zeatin	5	.09340	.032822	.014679	.05265	.13415	.057	.135
Total	87	.11172	.130313	.013971	.08395	.13950	.007	.882

Post Hoc Tests
Homogeneous Subsets

Weight

Duncan

Treatment	N	Subset for alpha = 0.05	
		1	2
0 (control)	6	.02750	
1.5 24d +1.5 Zeatin	7	.02929	
1.5 24d +1.5 Zeatin	8	.03412	
1.5 24d +1.5 Zeatin	5	.03560	
1.5 24d +1.5 Zeatin	4	.06625	
1.5 24d +1.5 Zeatin	5	.09340	
1.5 24d +1.5 Zeatin	4	.09900	
1.5 24d +1.5 Zeatin	5	.10180	
1.5 24d +1.5 Zeatin	6	.10417	
1.5 24d +1.5 Zeatin	3	.10667	
1.5 24d +1.5 Zeatin	5	.13000	
1.5 24d +1.5 Zeatin	6	.13650	.13650
1.5 24d +1.5 Zeatin	6	.13700	.13700
1.5 24d +1.5 Zeatin	3	.14533	.14533
Total	5	.18580	.18580
0 (control)	9		.29567
Sig.		.078	.053

Means for groups in homogeneous subsets are displayed.

TABLE A2: WEIGHT IN COMBINATION OF 2,4-D AND BAP (B)

Treatment		N	Mean weight of callus
BAP	2,4-D		Days 30 (Mean + S.E)
0	0	6	0.10 ± 0.08[a]
0	0	4	0.02 ± 0.00[a]
0	0	6	0.02 ± 0.00[a]
0.5	1.5	7	0.01 ± 0.00[a]
0.5	1.5	6	0.02 ± 0.01[a]
0.5	1.5	8	0.02 ± 0.00[a]
0.5	1.5	3	0.03 ± 0.01[a]
0.5	1.5	6	0.01 ± 0.00[a]
0.5	1.5	7	0.02 ± 0.00[a]
0.5	1.5	6	0.01 ± 0.00[a]
0.5	1.5	8	0.19 ± 0.09[a]
0.5	1.5	7	0.09 ± 0.02[a]
0.5	1.5	6	0.10 ± 0.02[a]
0.5	1.5	6	0.01 ± 0.00[a]
0.5	1.5	8	0.73 ± 0.21[b]
0.5	1.5	10	0.25 ± 0.09[a]

Result are mean ± SE of 16 treatment with 10 replicates each.
Means in a column followed by the same letter are not significantly different (P= 0.05) according to Duncan's multiple range test.

ANOVA

Weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.860	15	.191	6.751	.000
Within Groups	2.372	84	.028		
Total	5.232	99			

[DataSet1] D:\2,4D + BAP\SPSS Latest\Weight\weight.sav

Descriptives

Weight

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0 (control)	6	.1042	.19499	.07961	-.1005	.3088	.01	.50
0	4	.0152	.00450	.00225	.0081	.0224	.01	.02
0	6	.0182	.01326	.00541	.0043	.0321	.00	.04
1.5 24d + BAP	7	.0092	.00568	.00214	.0039	.0144	.00	.02
1.5 24d +BAP	6	.0235	.02093	.00854	.0015	.0455	.00	.06
1.5 24d +BAP	8	.0192	.00963	.00341	.0112	.0273	.01	.04
1.5 24d +BAP	3	.0277	.02479	.01431	-.0339	.0892	.01	.06
1.5 24d +BAP	6	.0118	.00172	.00070	.0100	.0136	.01	.01
1.5 24d +BAP	7	.1897	.24848	.09392	-.0401	.4195	.01	.60
1.5 24d +BAP	6	.0182	.01118	.00456	.0064	.0299	.00	.03
1.5 24d +BAP	8	.0180	.01014	.00359	.0095	.0265	.01	.04
1.5 24d +BAP	7	.0873	.06122	.02314	.0307	.1439	.00	.18
1.5 24d +BAP	6	.0987	.05577	.02277	.0401	.1572	.02	.16
1.5 24d +BAP	6	.0120	.00533	.00218	.0064	.0176	.00	.02
1.5 24d +BAP	6	.7310	.51174	.20892	.1940	1.2680	.00	1.41
1.5 24d +BAP	8	.1054	.25569	.09040	-.1084	.3191	.00	.74
Total	100	.0939	.22989	.02299	.0483	.1395	.00	1.41

Post Hoc Tests
Homogeneous Subsets
Weight

Duncan

Treatment	N	Subset for alpha = 0.05	
		1	2
1.5 24d + BAP	7	.0092	
1.5 24d +BAP	6	.0118	
1.5 24d +BAP	6	.0120	
1.5 24d +BAP	4	.0152	
1.5 24d +BAP	8	.0180	
1.5 24d +BAP	6	.0182	
1.5 24d +BAP	6	.0182	
1.5 24d +BAP	8	.0192	
1.5 24d +BAP	6	.0235	
1.5 24d +BAP	3	.0277	
1.5 24d +BAP	7	.0873	
1.5 24d +BAP	6	.0987	
0 (control)	6	.1042	
1.5 24d +BAP	8	.1054	
1.5 24d +BAP	7	.1897	
1.5 24d +BAP	6		.7310
Sig.		.137	1.000

Means for groups in homogeneous subsets are displayed.

TABLE A13: WEIGHT IN COMBINATION OF IBA AND 2,4-D (IBA)

Treatment		N	Mean weight of callus
2,4-D	IBA		Days 30 (Mean + S.E)
0	0	6	0.10 ± 0.08^{bc}
0	0	4	0.02 ± 0.00^{a}
0	0	6	0.02 ± 0.00^{a}
0	0.5	7	0.01 ± 0.00^{ab}
0	0.5	4	0.07 ± 0.02^{abc}
0	0.5	7	0.03 ± 0.00^{ab}
0	0.5	8	0.03 ± 0.00^{a}
0	0.5	4	0.02 ± 0.00^{ab}
0	0.5	6	0.03 ± 0.01^{ab}
0	0.5	4	0.03 ± 0.01^{ab}
0	0.5	4	0.14 ± 0.04^{c}
0	0.5	7	0.09 ± 0.02^{abc}
0	0.5	3	0.05 ± 0.01^{ab}
0	0.5	4	0.02 ± 0.00^{a}
0	0.5	6	0.01 ± 0.00^{a}
0	0.5	9	0.18 ± 0.00^{a}

Result are mean ± SE of 16 treatment with 10 replicates each.
Means in a column followed by the same letter are not significantly different (P= 0.05) according to Duncan's multiple range test.

ANOVA

Weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.113	15	.008	2.247	.012
Within Groups	.245	73	.003		
Total	.358	88			

[DataSet1] D:\2,4d + iba\SPSS Latest\Weight\weight.sav

Descriptives

Weight

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0 (control)	6	.10417	.194995	.079606	-.10047	.30880	.007	.500
iba	4	.01525	.004500	.002250	.00809	.02241	.010	.021
iba	6	.01817	.013258	.005412	.00425	.03208	.005	.042
iba	7	.00916	.005675	.002145	.00391	.01441	.001	.019
iba	4	.06625	.045529	.022765	-.00620	.13870	.021	.113
iba	7	.02929	.008261	.003122	.02165	.03693	.018	.041
iba	8	.03412	.005515	.001950	.02951	.03874	.027	.042
iba	4	.01950	.010599	.005299	.00264	.03636	.010	.031
iba	6	.03350	.022757	.009291	.00962	.05738	.018	.068
iba	4	.03125	.017951	.008976	.00269	.05981	.018	.057
iba	4	.14325	.078568	.039284	.01823	.26827	.089	.256
iba	7	.08729	.061223	.023140	.03066	.14391	.000	.181
iba	3	.05133	.020502	.011837	.00040	.10226	.031	.072
iba	4	.01700	.008756	.004378	.00307	.03093	.008	.029
iba	6	.01250	.006626	.002705	.00555	.01945	.004	.024
iba	9	.01656	.006673	.002224	.01143	.02168	.009	.029
Total	89	.04086	.063747	.006757	.02743	.05428	.000	.500

Post Hoc Tests
Homogeneous Subsets

Weight

Duncan

Treatment	N	Subset for alpha = 0.05		
		1	2	3
iba	7	.00916		
iba	6	.01250		
iba	4	.01525		
iba	9	.01656		
iba	4	.01700		
iba	6	.01817		
iba	4	.01950	.01950	
iba	7	.02929	.02929	
iba	4	.03125	.03125	
iba	6	.03350	.03350	
iba	8	.03412	.03412	
iba	3	.05133	.05133	
iba	4	.06625	.06625	.06625
iba	7	.08729	.08729	.08729
0 (control)	6		.10417	.10417
iba	4			.14325
Sig.		.081	.051	.056

Means for groups in homogeneous subsets are displayed.

TABLE A20: WEIGHT IN OF 2,4D

Treatment		N	Mean weight of callus
2,4D	2,4D		Days 30 (Mean + S.E)
1.5	0	6	0.10 ± 0.08^{abc}
1.5	0	4	0.07 ± 0.02^{ab}
1.5	0	7	0.03 ± 0.00^{a}
1.5	0	8	0.26 ± 0.00^{a}
1.5	0	4	0.07 ± 0.02^{ab}
1.5	0	7	0.03 ± 0.00^{a}
1.5	0	8	0.03 ± 0.00^{a}
1.5	0	5	0.13 ± 0.02^{abc}
1.5	0	9	0.13 ± 0.03^{abc}
1.5	0	7	0.20 ± 0.01^{c}
1.5	0	5	0.16 ± 0.01^{ab}
1.5	0	5	0.13 ± 0.06^{abc}
1.5	0	10	0.05 ± 0.01^{ab}
1.5	0	6	0.13 ± 0.03^{abc}
1.5	0	5	0.19 ± 0.06^{c}
1.5	0	6	0.16 ± 0.07^{bc}

Result are mean ± SE of 16 treatment with 10 replicates each.
Means in a column followed by the same letter are not significantly different (P= 0.05) according to Duncan's multiple range test.

ANOVA

Weight					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.332	15	.022	3.150	.000
Within Groups	.604	86	.007		
Total	.937	101			

[DataSet1] D:\2,4d + 2,4d \SPSS Latest\Weight\weight.sav

Descriptives

Weight

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0 (control)	6	.10417	.194995	.079606	-.10047	.30880	.007	.500
24d	4	.06625	.045529	.022765	-.00620	.13870	.021	.113
24d	7	.02929	.008261	.003122	.02165	.03693	.018	.041
24d	8	.03412	.005515	.001950	.02951	.03874	.027	.042
24d	4	.06625	.045529	.022765	-.00620	.13870	.021	.113
24d	7	.02929	.008261	.003122	.02165	.03693	.018	.041
24d	8	.03412	.005515	.001950	.02951	.03874	.027	.042
24d	5	.12620	.049505	.022139	.06473	.18767	.082	.211
24d	9	.12978	.078190	.026063	.06968	.18988	.033	.247
24d	7	.20143	.043768	.016543	.16095	.24191	.147	.264
24d	5	.07560	.036508	.016327	.03027	.12093	.031	.132
24d	5	.13080	.131768	.058928	-.03281	.29441	.031	.312
24d	10	.04980	.028701	.009076	.02927	.07033	.021	.120
24d	6	.13317	.081994	.033474	.04712	.21921	.032	.266
24d	5	.19460	.134292	.060057	.02785	.36135	.073	.406
24d	6	.15733	.168824	.068922	-.01984	.33450	.036	.443
Total	102	.09378	.096297	.009535	.07487	.11270	.007	.500

Post Hoc Tests
Homogeneous Subsets

Weight

Duncan

Treatment	N	Subset for alpha = 0.05		
		1	2	3
24d	7	.02929		
24d	7	.02929		
24d	8	.03412		
24d	8	.03412		
24d	10	.04980	.04980	
24d	4	.06625	.06625	
24d	4	.06625	.06625	
24d	5	.07560	.07560	
0 (control)	6	.10417	.10417	.10417
24d	5	.12620	.12620	.12620
24d	9	.12978	.12978	.12978
24d	5	.13080	.13080	.13080
24d	6	.13317	.13317	.13317
24d	6		.15733	.15733
24d	5			.19460
24d	7			.20143
Sig.		.081	.066	.091

Means for groups in homogeneous subsets are displayed.

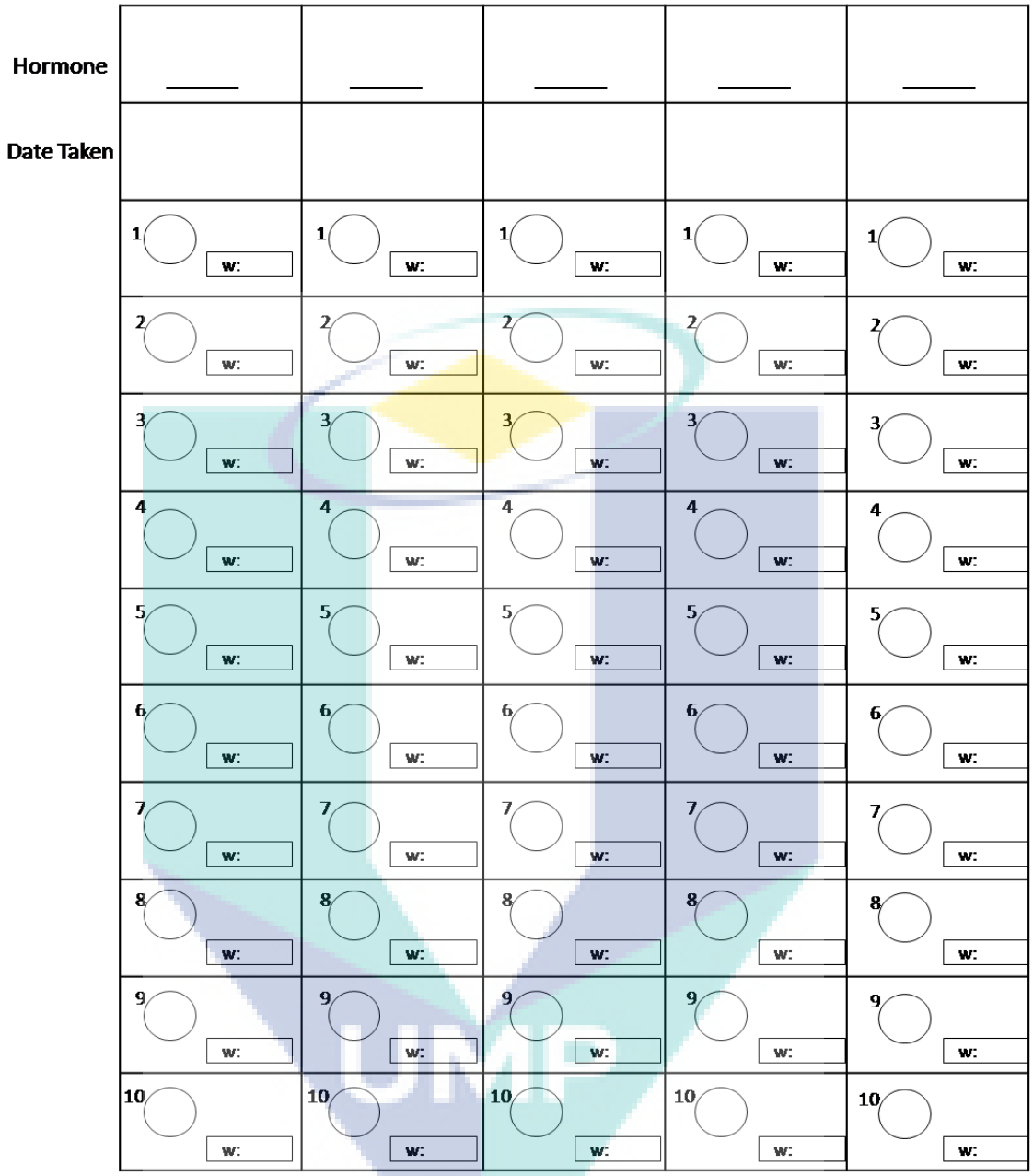


Figure B2: Observation form of weight

T-TEST CALLUS INDUCTION Z

[DataSet0] C:\Users\ASUS\Documents\callusinduction.sav

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
z	60	.8000	.40338	.05208

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
z	15.362	59	.000	.80000	.6958	.9042

T-Test callus induction B

[DataSet0] C:\Users\ASUS\Documents\callusinduction.sav

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
b	60	.8667	.34280	.04426

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
b	19.583	59	.000	.86667	.7781	.9552

T-TEST CALLUS INDUCTION IBA

[DataSet1] C:\Users\ASUS\Desktop\spsscallusinduction\tiba.sav

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
iba	60	.6500	.48099	.06210

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
iba	10.468	59	.000	.65000	.5257	.7743

T-TEST CALLUS INDUCTION 2,4D 1

[DataSet1] C:\Users\ASUS\Desktop\spsscallusinduction\24d1.sav

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
d1	60	.2833	.45442	.05867

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
d1	4.830	59	.000	.28333	.1659	.4007

T-TEST CALLUS INDUCTION 2,4D 2

[DataSet0] C:\Users\ASUS\Documents\callusinduction 2,4D 2.sav

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
d2	60	.6833	.46910	.06056

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
d2	11.283	59	.000	.68333	.5622	.8045

T-TEST CALLUS INDUCTION 2,4D 3

[DataSet0] C:\Users\ASUS\Documents\callusinduction24d3.sav

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
d3	60	.8833	.32373	.04179

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
d3	21.136	59	.000	.88333	.7997	.9670

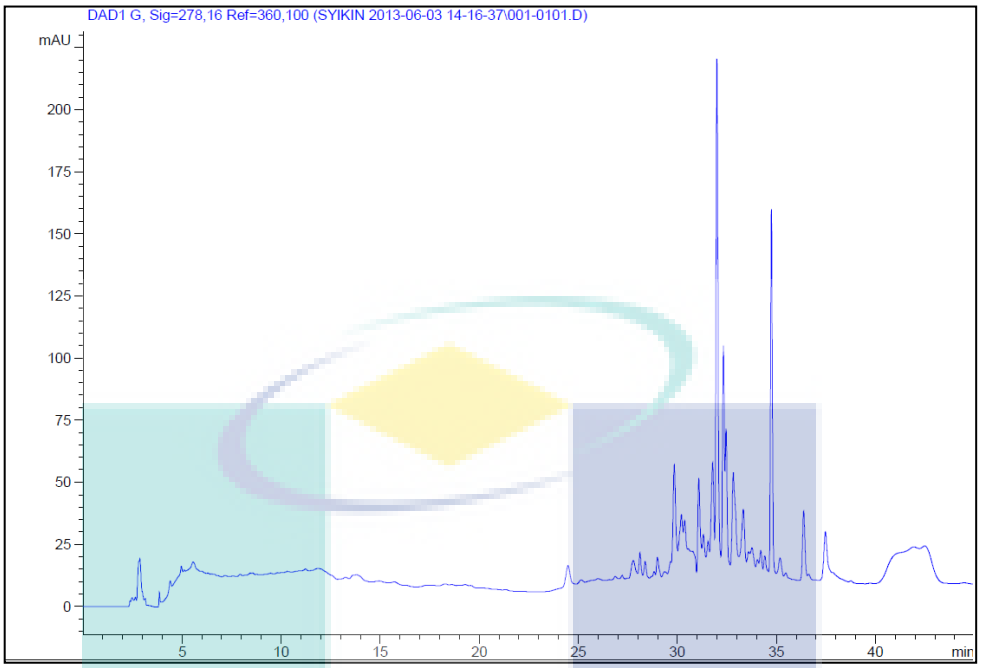


Figure 4.8.2: HPLC chromatogram containing callus extract in MS media treated with 1.5 mg/L Zeatin + 1.5 mg/L 2,4-D (λ= 278nm)

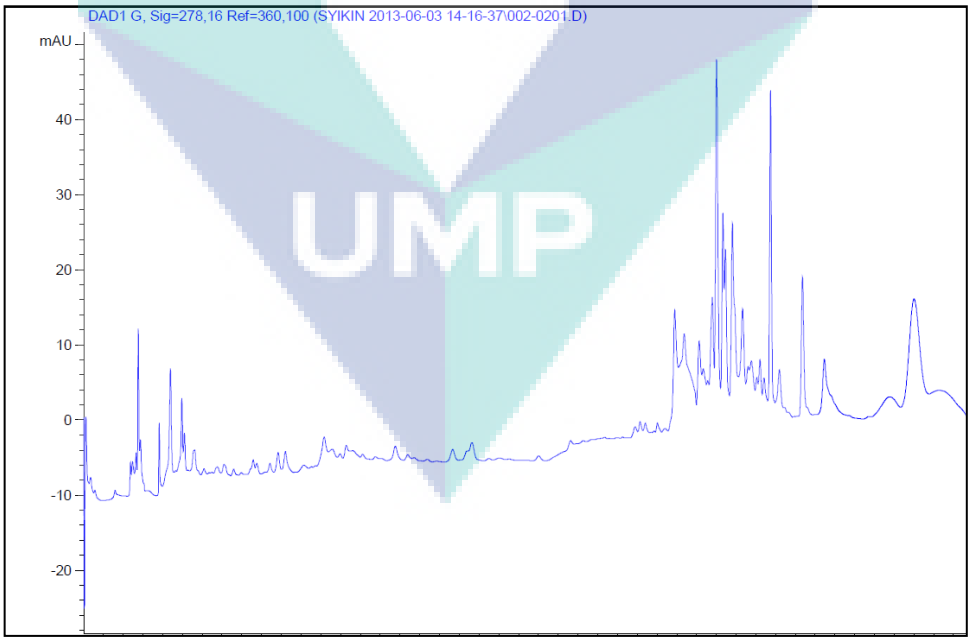


Figure 4.8.3: HPLC chromatogram containing callus extract in MS media treated with 1.5 mg/L BAP + 1.5 mg/L 2,4-D (λ= 278nm)

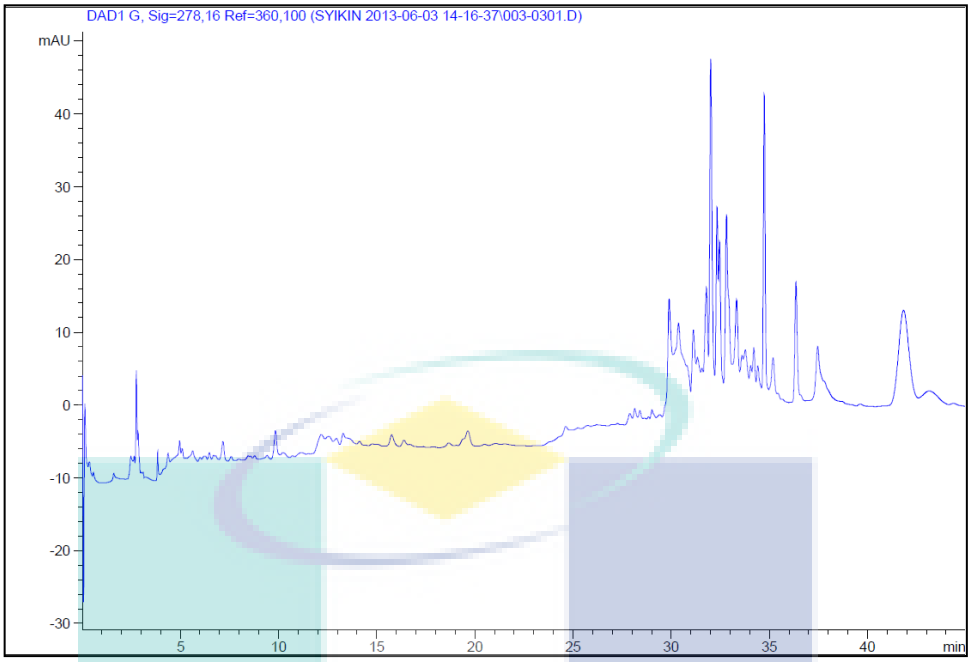


Figure 4.8.4: HPLC chromatogram containing callus extract in MS media treated with 1.5 mg/L IBA (λ= 278nm)

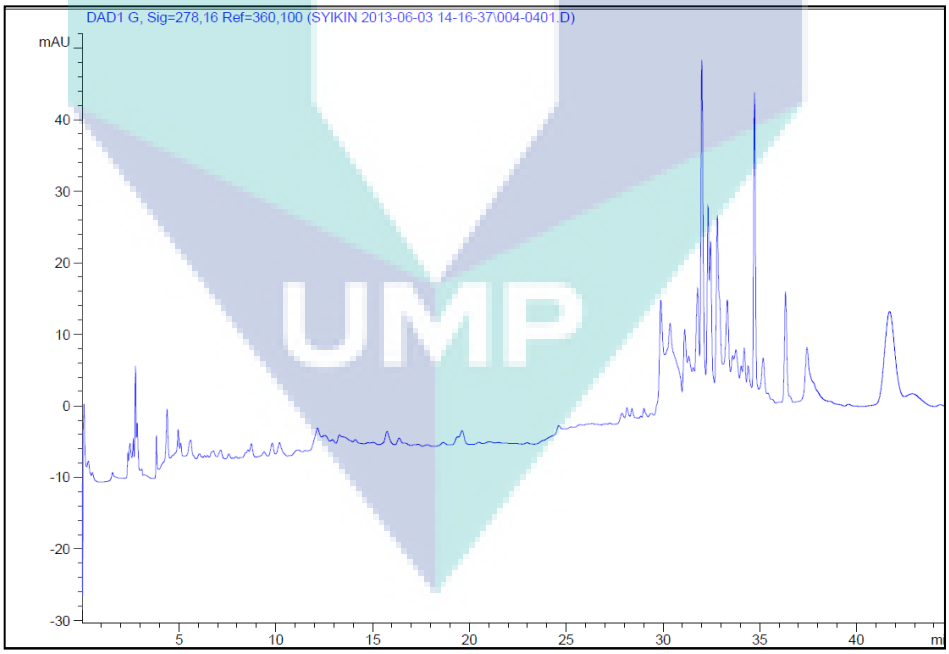


Figure 4.8.5: HPLC chromatogram containing callus extract labelled as 2,4-D (λ= 278nm)

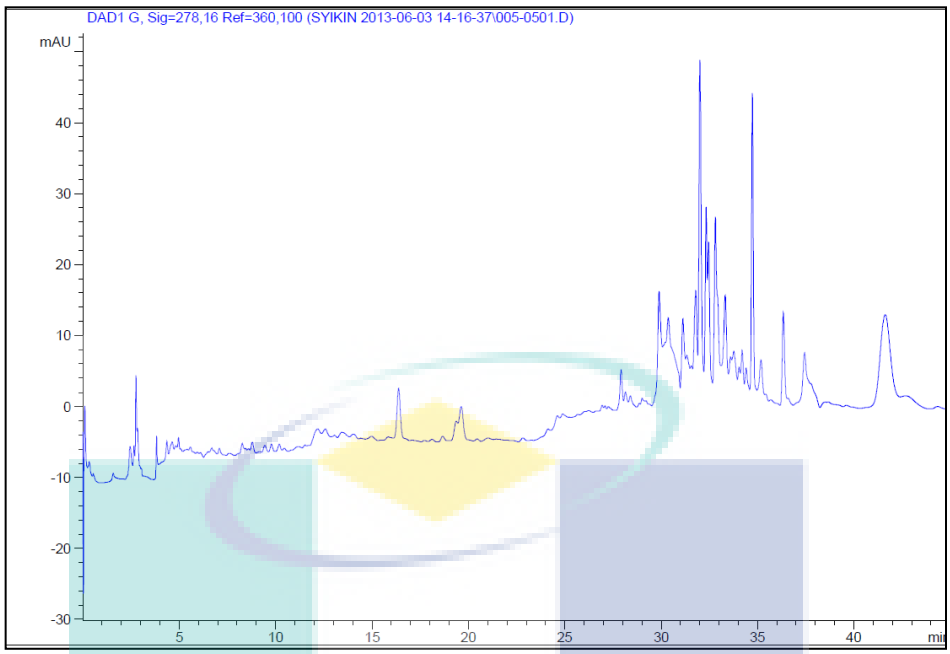


Figure 4.8.6: HPLC chromatogram containing leaf extract (λ = 278nm)

Table A21: Availability of GA and CE in the extracts

Extract	*GA (RT: 5.0-5.278 min) (λ= 278) nm*	*Abundance (%)*	*CE (RT: 7.0-7.836 min) (λ= 278) nm*	*Abundance (%)*
Zeatin	5.109	1.1302	-	
BAP	5.096	1.1605	7.589	0.5268
IBA	5.096	0.2492	7.170	0.4742
2,4D	5.097	0.6848	7.161	0.2254
Leaf	-	-	-	-

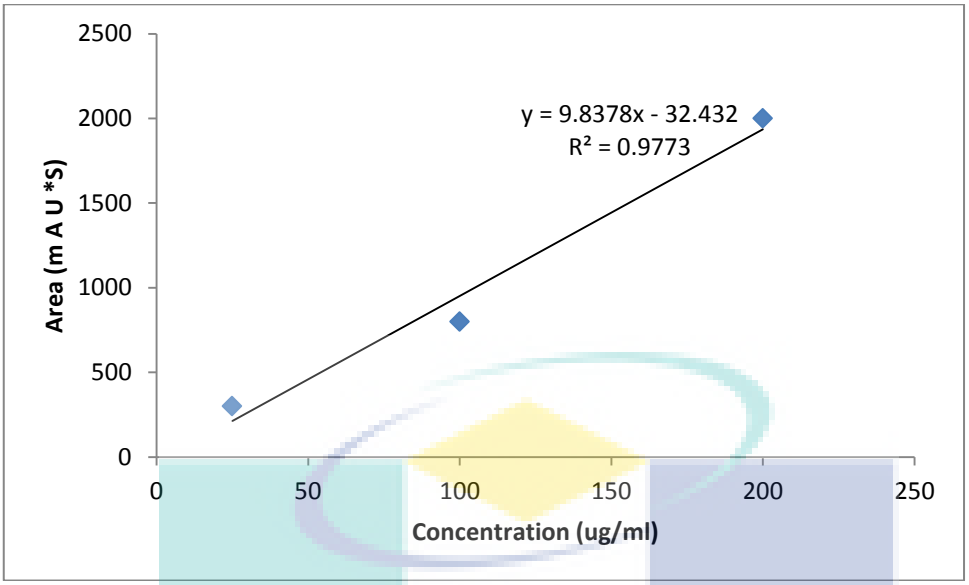


Figure A 1 Gallic acid (HPLC)

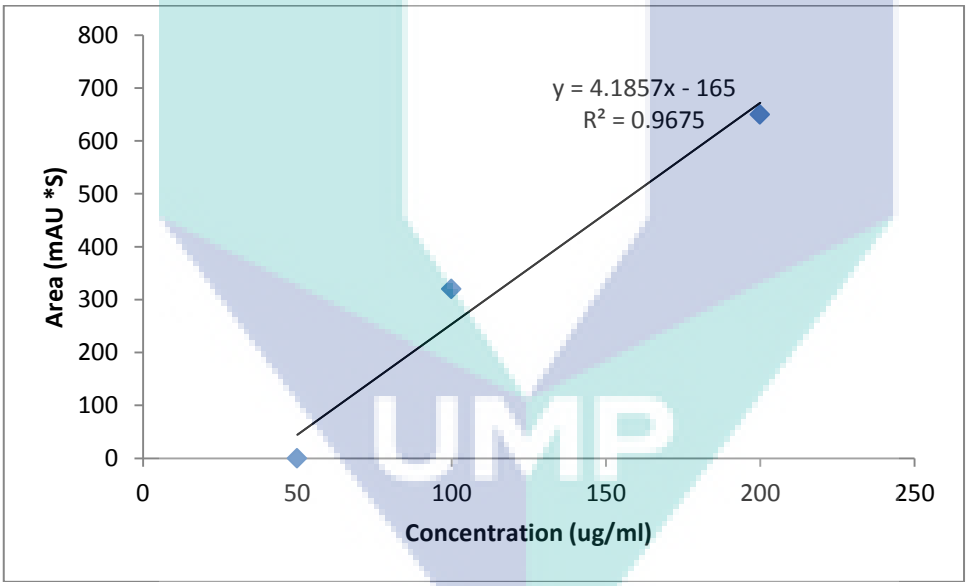


Figure A 2Catechin hydrate (HPLC)

ANTIBACTERIAL TEST- DISK DIFFUSION

S. Aureus

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
z	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
B	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
IBA	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
D	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
Control	Between Groups	114.000	2	57.000	171.000	.000
	Within Groups	2.000	6	.333		
	Total	116.000	8			

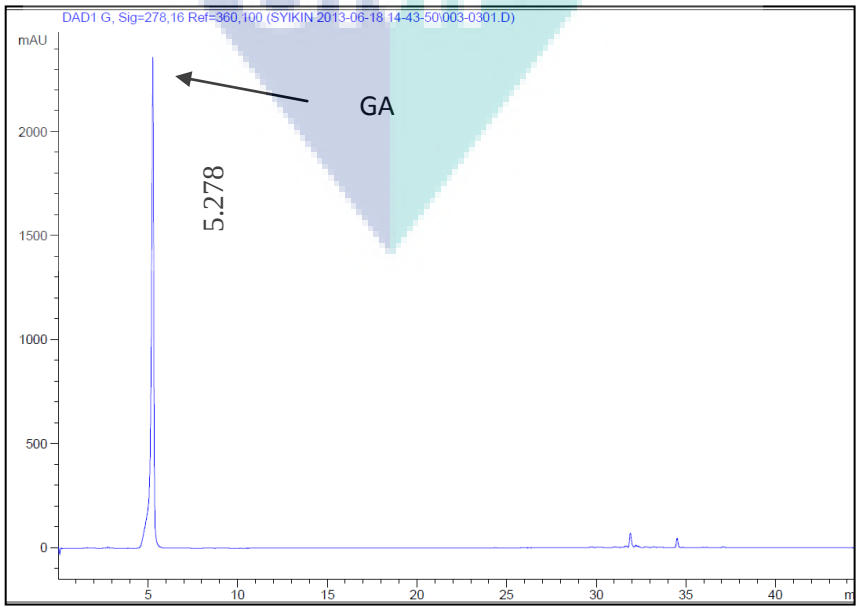


Figure 4.8: HPLC chromatogram containing Gallic acid (200 µg/ml). Retention time (5.278 min) (λ= 278nm)

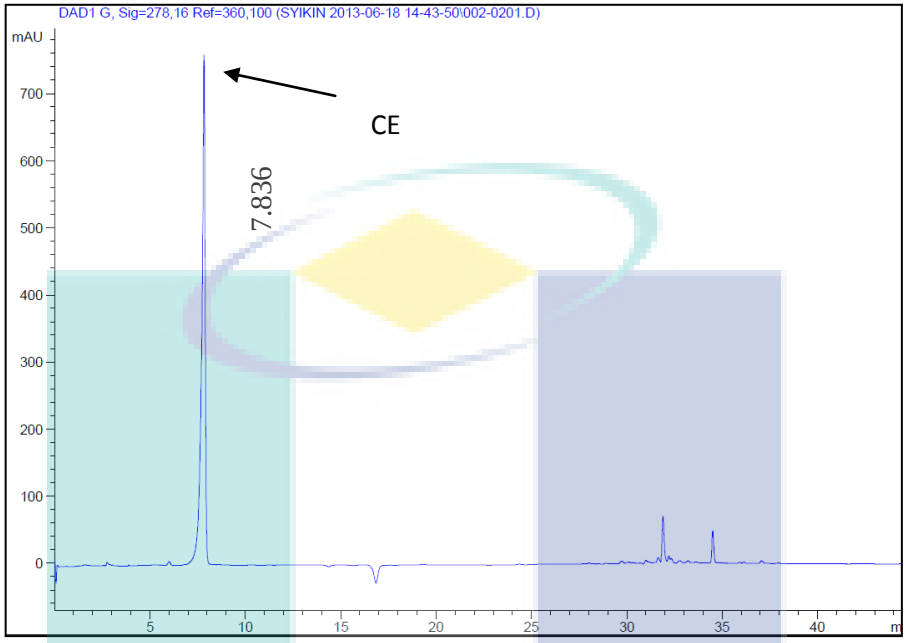


Figure 4.8.1: HPLC chromatogram containing Catechin hydrate (200 µg/ml). Retention time (7.836) (λ= 278nm)

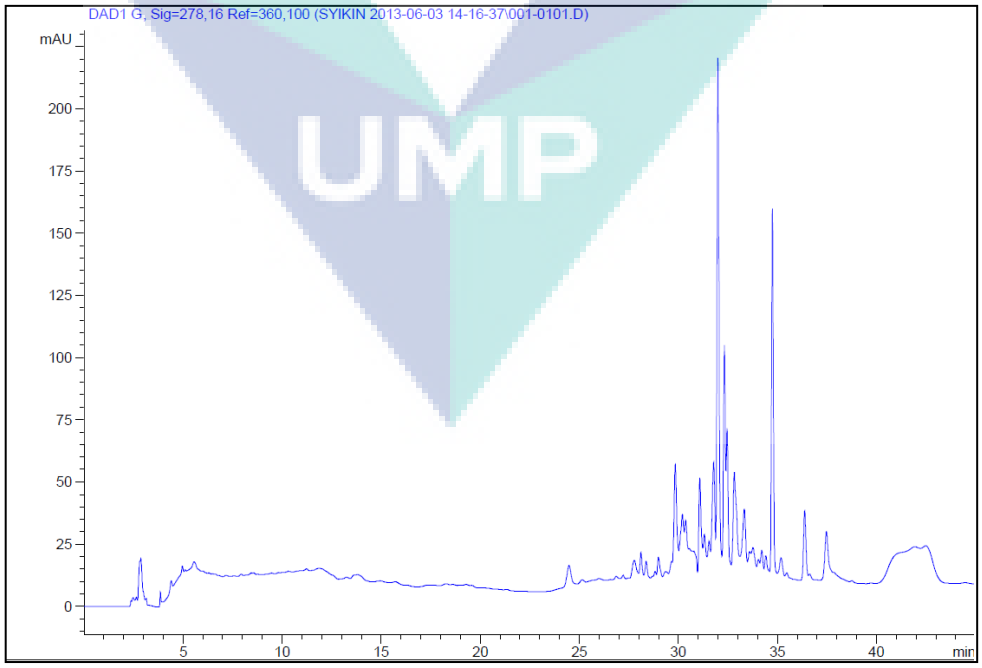


Figure 4.8.2: HPLC chromatogram containing callus extract in MS media treated with 1.5 mg/L Zeatin + 1.5 mg/L 2,4-D (λ= 278nm)

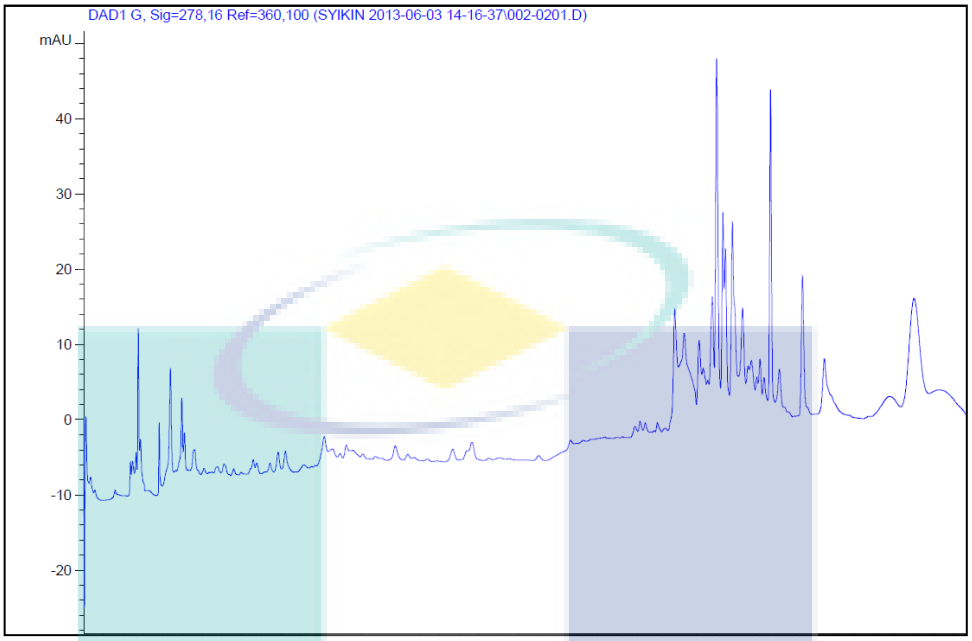


Figure 4.8.3: HPLC chromatogram containing callus extract in MS media treated with 1.5 mg/L BAP + 1.5 mg/L 2,4-D (λ= 278nm)

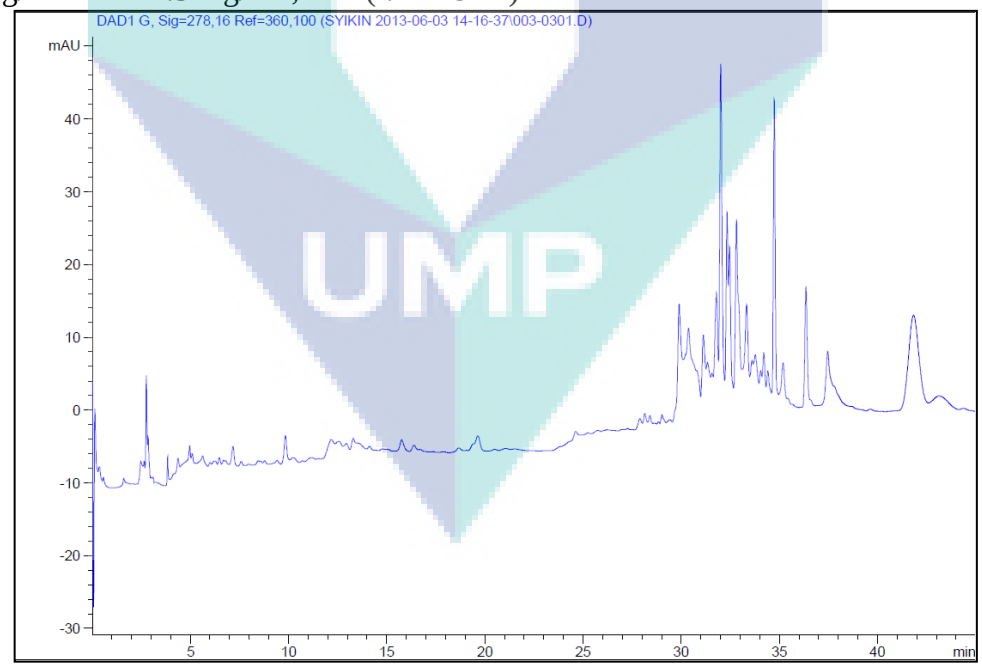


Figure 4.8.4: HPLC chromatogram containing callus extract in MS media treated with 1.5 mg/L IBA (λ= 278nm)

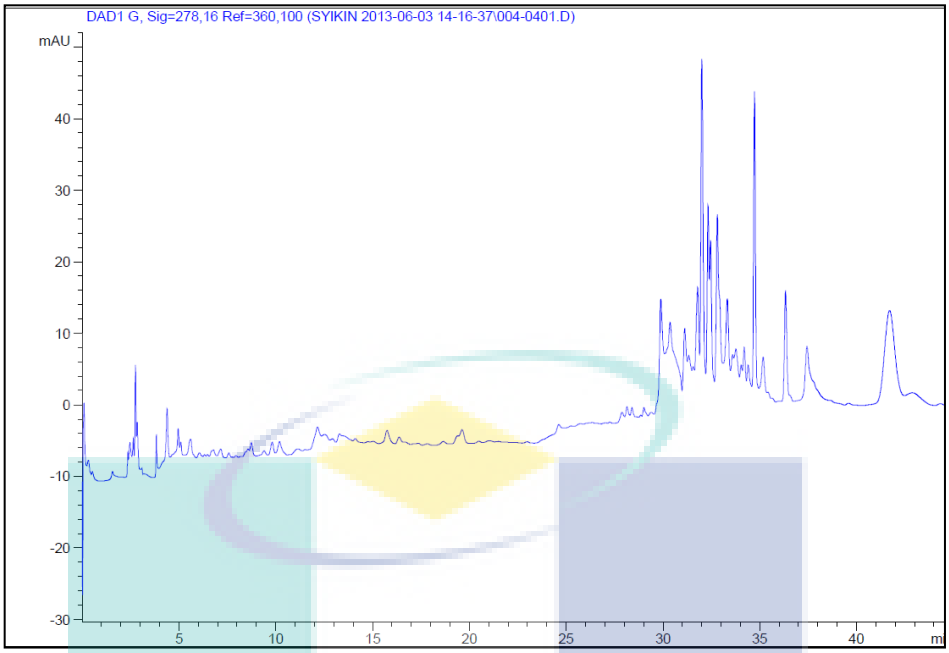


Figure 4.8.5: HPLC chromatogram containing callus extract labelled as 2,4-D (λ= 278nm)

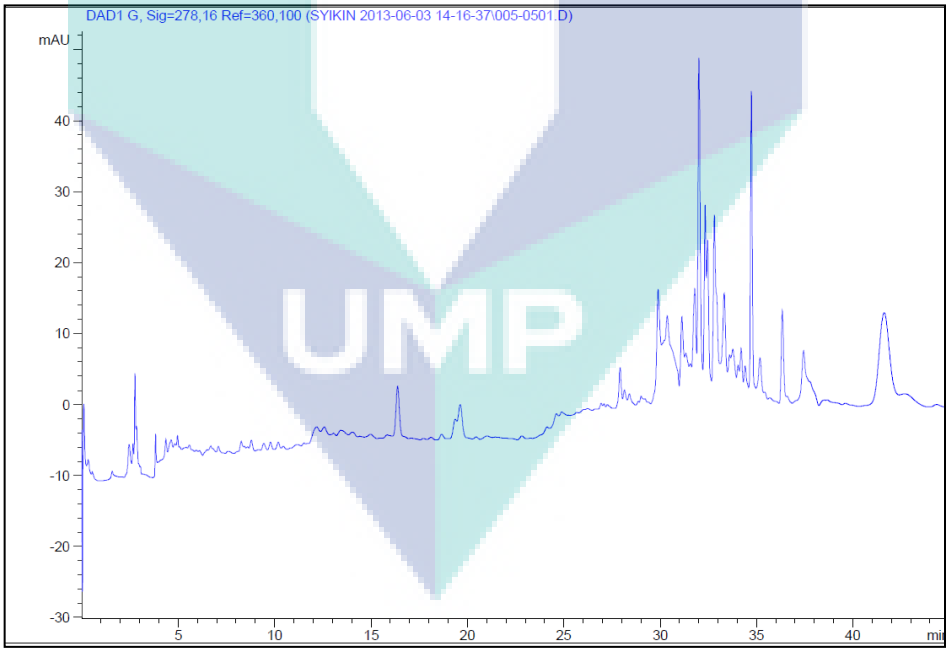


Figure 4.8.6: HPLC chromatogram containing leaf extract (λ = 278nm)

Table 4.6: Availability of GA and CE in the extracts

Extract	*GA (RT: 5.0-5.278 min) (λ= 278) nm*	*Abundance (%)*	*CE (RT: 7.0-7.836 min) (λ= 278) nm*	*Abundance (%)*
Zeatin	5.109	1.1302	-	
BAP	5.096	1.1605	7.589	0.5268
IBA	5.096	0.2492	7.170	0.4742
2,4D	5.097	0.6848	7.161	0.2254
Leaf	-	-	-	-

P.Aeroginosa

```
[DataSet0] C:\Users\ASUS\Documents\paeroginosa.sav
```

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
z	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
B	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
IBA	Between Groups	648.000	2	324.000	.	.
	Within Groups	.000	6	.000		
	Total	648.000	8			
D	Between Groups	600.000	2	300.000	.	.
	Within Groups	.000	6	.000		
	Total	600.000	8			
Control	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			

B. Subtilis

[DataSet0] C:\Users\ASUS\Documents\bsubtilis.sav

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
z	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
B	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
IBA	Between Groups	150.889	2	75.444	75.444	.000
	Within Groups	6.000	6	1.000		
	Total	156.889	8			
D	Between Groups	600.000	2	300.000	.	.
	Within Groups	.000	6	.000		
	Total	600.000	8			
Control	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
IBA	1	2	-5.66667*	.81650	.001	-8.1719	-3.1614
		3	-10.00000*	.81650	.000	-12.5052	-7.4948
	2	1	5.66667*	.81650	.001	3.1614	8.1719
		3	-4.33333*	.81650	.004	-6.8386	-1.8281
	3	1	10.00000*	.81650	.000	7.4948	12.5052
		2	4.33333*	.81650	.004	1.8281	6.8386

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

IBA

Tukey HSD

VAR00001	N	Subset for alpha = 0.05		
		1	2	3
1	3	14.6667		
2	3		20.3333	
3	3			24.6667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

E coli

[DataSet0] C:\Users\ASUS\Documents\ecoli.sav

ONE WAY ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
z	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
B	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
IBA	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
D	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
Control	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			

S. typhii

[DataSet0] C:\Users\ASUS\Documents\ecoli.sav

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
z	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
B	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
IBA	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
D	Between Groups	150.000	2	75.000	.	.
	Within Groups	.000	6	.000		
	Total	150.000	8			
Control	Between Groups	.000	2	.000	.	.
	Within Groups	.000	5	.000		
	Total	.000	7			

E.feccalis

[DataSet0] C:\Users\ASUS\Documents\styphidisk.sav

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
z	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
B	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
IBA	Between Groups	600.000	2	300.000	.	.
	Within Groups	.000	6	.000		
	Total	600.000	8			
D	Between Groups	150.000	2	75.000	.	.
	Within Groups	.000	6	.000		
	Total	150.000	8			
Control	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			

ACE inhibitory activity	**Blank 1**	0.679	1.019	0.756	**0.818**
	Blank 2	0.076	0.069	0.068	**0.071**

	Abs 1	%	Abs 2	%	Abs 3	%	Stdev	Average	Table
Z	0.305	68.6747	0.341	63.85542	0.386	57.83133	5.432831	63.45382	63.45 ± 5.43
B	0.389	57.42972	0.403	55.55556	0.405	55.28782	1.167041	56.09103	56.09 ± 1.16
I	0.425	52.61044	0.442	50.33467	0.449	49.39759	1.652254	50.7809	50.78 ± 1.65
2,4	0.287	71.08434	0.291	70.54886	0.3	69.34404	0.891342	70.32575	70.32 ± 0.89
LE	0.309	68.13922	0.3	69.34404	0.289	70.8166	1.340917	69.43329	69.43 ± 1.34
Captopril	0.131	91.96787	0.104	95.58233	0.126	92.63722	1.922933	93.39581	93.39 ± 1.92

ACE Inhibitory

[DataSet0] C:\Users\ASUS\Documents\antihypertensivezbiba.sav

One way ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Z	Between Groups	59.001	2	29.500	.	.
	Within Groups	.000	0	.		
	Total	59.001	2			
B	Between Groups	2.879	2	1.440	.	.
	Within Groups	.000	0	.		
	Total	2.879	2			
IBA	Between Groups	5.456	2	2.728	.	.
	Within Groups	.000	0	.		
	Total	5.456	2			
D	Between Groups	1.591	2	.795	.	.
	Within Groups	.000	0	.		
	Total	1.591	2			
L	Between Groups	3.604	2	1.802	.	.
	Within Groups	.000	0	.		
	Total	3.604	2			

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  /PRINT=TWOTAIL NOSIG
  /STATISTICS DESCRIPTIVES

  /MISSING=PAIRWISE.
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Correlations

Notes

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TFC1	73.2850	68.79666	4
Antihypertensive1	60.1600	8.53618	4

Correlations

		TFC1	Antihypertensive 1
TFC1	Pearson Correlation	1	-.851
	Sig. (2-tailed)		.149
	N	4	4
Antihypertensive1	Pearson Correlation	-.851	1
	Sig. (2-tailed)	.149	
	N	4	4

Correlations

Notes

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	Cases Used	Statistics for each pair of variables are based on all the cases with valid data for that pair.
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Descriptive Statistics

	Mean	Std. Deviation	N
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Antibacterial1	.7500	.95743	4

Correlations

		TFC1	Antibacterial1
TFC1	Pearson Correlation	1	-.340
	Sig. (2-tailed)		.660
	N	4	4
Antibacterial1	Pearson Correlation	-.340	1
	Sig. (2-tailed)	.660	
	N	4	4

Correlation

Notes

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Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each pair of variables are based on all the cases with valid data for that pair.
Syntax		CORRELATIONS /VARIABLES=TPC1 Antihypertensive1 /PRINT=TWOTAIL NOSIG /STATISTICS DESCRIPTIVES /MISSING=PAIRWISE.
Resources	Processor Time	00:00:00.063
	Elapsed Time	00:00:00.053

[DataSet0]

Descriptive Statistics

	Mean	Std. Deviation	N
TPC1	1.6742E2	103.78748	4
Antihypertensive1	60.1600	8.53618	4

Correlations

		TPC1	Antihypertensive1
TPC1	Pearson Correlation	1	.611
	Sig. (2-tailed)		.389
	N	4	4
Antihypertensive1	Pearson Correlation	.611	1
	Sig. (2-tailed)	.389	
	N	4	4

Correlations

Notes

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	Cases Used	Statistics for each pair of variables are based on all the cases with valid data for that pair.
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Descriptive Statistics

	Mean	Std. Deviation	N
TPC1	1.6742E2	103.78748	4
Antibacterial1	.7500	.95743	4

Correlations

		TPC1	Antibacterial1
TPC1	Pearson Correlation	1	.966*
	Sig. (2-tailed)		.034
	N	4	4
Antibacterial1	Pearson Correlation	.966*	1
	Sig. (2-tailed)	.034	
	N	4	4

*. Correlation is significant at the 0.05 level (2-tailed).