UNIVERSITI PUTRA MALAYSIA

THE PRODUCTION OF FLAVONOIDS (QUERCETIN AND HESPERETIN) FROM CALLUS CULTURE OF *Citrus aurantifolia* (CHRISTM & PANZER) SWINGLE

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By

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Thesis Submitted In Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies Universiti Putra Malaysia

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LIST OF ABBREVIATION

HPLC - high performance liquid chomatography

µg - microgram

g - gram

ml - milliliter

g. fr. wt. - gram fresh weight

g. d. wt. - gram dry weight

2,4-D - 2,4 dichlorophenoxyacetic acid

BAP - benzylamino purine

NAA - α-naphthaleneacetic acid

kinetin - 6-furfurilamino purine

TTC - 2,3,5-triphenyl tetrazolium chloride

MS medium - Murashige and Skoog Medium

i.e. - that is

nm - nanometer

cm - centimeter

UV - ultra violet

(v/v) - volume/volume

(w/v) - weight/volume

NA - nutrient agar

NB - nutrient broth

LiCl - Lithium Chlorida

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman : Associate Professor Radzali Muse Ph.D.

Faculty : Science and Environmental Studies

The application of plant tissue culture technique for plant flavonoids production shows some promising results. However, certain limitation exist mainly because many cultures do not produce significant amount of the characteristic compounds from the plants which they were derived. Thus, the objectives of this study were to establish the *C. aurantifolia* (Christm & Panzer) Swingle callus cultures for bioproduction system of flavonoid compounds (quercetin and hesperetin) and to optimize their production by application of yeast extract supplementation.

The callus culture was established by planting the explant excised from peel of mature fruit on the basal medium Murashige and Skoog (MS) (1962) supplemented with 1.5 mg/L (w/v) α-naphthalene acetic acid (NAA),

0.5 mg/L (w/v) 6-furfurylamino purine (kinetin), 30 g/L (w/v) sucrose and solidified with 3.0 g/L (w/v) phytagel (pH medium 5.7). Growth of callus cultures incubated in the light and dark conditions at 27 ± 2 °C showed similar sigmoidal patterns, in which their maximum growth at 6th week of incubation reached 314% and 264% respectively. The major flavonoid compounds in callus cultures were determined using HPLC technique with UV detector. It was found that the callus cultures could produce the major flavonoids quercetin and hesperetin. The quercetin production from callus incubated in the light and dark condition was 19.73 µg/g. d. wt. tissue and 1.90 µg/g. d. wt. tissue respectively whilst the hesperitin production from callus incubated in the light and dark conditions was 0.64 µg/g.d.wt. tissue and 0.58 µg/g.d.wt. tissue respectively. Supplementation of relatively low concentration of yeast extract (YE) (0.5, 1.0 and 2.0 g/L, (w/v)) to the culture medium did not show any effect on the quercetin and hesperitin production from callus cultures. Higher concentration of yeast extract (YE) supplementation (4.0 g/L, (w/v)) was found to decrease the production of quercetin and hesperitin by 18.92% and 7.82% for the callus incubated in the light condition 13.56% and 6.54% for the callus incubated in the dark condition. This results indicated that yeast extract was not a suitable elicitor for enhancing the production of quercetin and hesperetin from *C. aurantifolia* callus cultures.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENGHASILAN FLAVONOIDS (KUARSETIN DAN HESPERETIN) DARIPADA KULTUR KALUS *Citrus aurantifolia* (CHRISTM & PANZER) SWINGLE

Oleh

SITI MAHYUNI

Julai 1999

Pengerusi : Profesor Madya Radzali Muse, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Penghasilan flavonoids dengan menggunakan teknik kultur tisu telah menampakkan hasil-hasil yang memberangsangkan. Walau bagaimanapun terdapat faktor penghad di dalam penghasilan ini karena kebanyakan kultur tidak memberikan hasil yang signifikan. Maka, objektif kajian ini ialah untuk menghasilkan kultur tisu *C aurantifolia* (Christm & Panzer) Swingle bagi penghasilan sebatian flavonoids (kuarsetin dan hesperetin) dan untuk mengoptimalkan penghasilan dengan penambahan ekstrak yis.

Kultur kalus dihasilkan dengan meletakan eksplan kulit buah matang pada media asas medium asas Murashige dan Skoog (1962) (pH 5.7) dengan penambahan 1.5 mg/L (b/i) asid α-naftalena asetik (NAA), 0.5 mg/L

(b/i) kinetin, 30 g/L (b/i) sukrosa dan telah dibekukan dengan pemberian 3.0 g/L (b/i) phytagel. Kultur ini telah dieramkan dalam keadaan gelap dan terang pada suhu 27 ± 2 °C, dan menunjukan suatu bentuk sigmoid yang serupa dimana pertumbuhan maksima pada minggu ke-6 pengeraman masing-masing mencapai 314% dan 264%.

Sebatian flavonoid utama pada kultur kalus telah dapat ditentukan menggunakkan teknik HPLC dengan suatu alat pengesan ultra ungu (UV). lanya didapati kultur kalus boleh menghasilkan flavonoid utama, kuarsetin dan hesperetin. Penghasilan kuarsetin daripada kalus yang telah dieramkan di dalam keadaan terang dan gelap adalah masing-masing 19.73μg/g.bt. kering dan 1.9 μg/g.bt. kering tisu manakala penghasilan hesperetin di dalam keadaan terang dan gelap masing-masing 0.64 μg/g.bt. kering dan 0.58 μg/g.bt. kering tisu.

Penambahan ekstrak Yis yang berkepekatan rendah (0.5, 1.0, dan 2.0, g/L, (b/i)) tidak memberi kesan apa-apa terhadap penghasilan kuarsetin dan hesperetin dari kultur kalus tetapi kepekatan penambahan ekstrak yis yang tinggi (4.0 g/L (b/i)) boleh menyebabkan penghasilan kuarsetin dan hesperetin masing-masing berkurang kepada 18.92% dan 7.82%. Hasil kajian ini menunjukkan bahwa ekstrak yis bukan elisitor yang sesuai untuk menggalakkan penghasilan kuarsetin dan hesperetin daripada kultur kalus *C. aurantifolia*.

CHAPTER I

INTRODUCTION

Flavonoids have many valuable properties in plant biochemistry and plant physiology, i.e. as antioxidants, enzyme inhibitors, precursor of toxic substances, natural pigments and UV light screener. In addition, these compounds are involved in a wide spectrum of activities in plant photosynthesis, energy transfer, actions of plant growth hormones, natural growth regulators, morphogenesis, sex determination and defense against infections (Harbone, 1994). Recent reports indicated that plant flavonoids activate bacterial nodulation genes involving in the control of nitrogen fixation in root nodules of legumes and *Casuarina spp.*, which suggests it's having important relationships between particular flavonoids and the activation and expressions of genes (Harbone and Mabry, 1982).

Flavonoids are well known common constituents of many medicinal plants and herbs. They play an important role in biochemical and physiological functions of animal and human life. Some of them inhibit a series of enzyme system including hydrolase, ATPases, cAMPphospho-diesterases, kinases, lipases and transferases. They also have many

biological activities including anti-allergic, anti-cancer, anti-inflammatory, anti-hepatotoxic, anti-microbial, anti-ulcer, anti-viral, spasmolytic and become a potential for new drugs derived from the higher plants (Philipson, 1990).

Flavonoids have a widespread distribution in all part of the higher plants. Over 4000 chemically unique flavonoids have been identified in plant sources. These low molecular weight substances found in all vascular plants, which are phenylbenzopyrones (phenylchromones) with an assortment of basic structures. Primarily, they were recognized as the natural pigments responsible for the autumnal burst of the hues and many shades of yellow, orange, and red in flowers and foods. Natural flavonoids are found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea and wine and are important constituents of human diet. They are prominent components of citrus fruits and other food sources. On average, the daily western people diet contains approximately 1 gram of mixed flavonoids, a quantity that could provide pharmacologically significant concentration in body fluids and tissues (Hermann, 1976 and Kahnou, 1976).

Rutaceae, as a big family of the higher plant is extremely versatile in its synthetic capacity and produces a wide range of unusual and highly substitued flavonoid constituents (Cody, 1988). These plants have been

used in traditional medicine for e.g *Aegle marmaleos* is used as an agent for laxative, diuretic, cardiac depressant opthalmia and other eye infections. In tropical country, the root of *Murraya paniculata* is used to cure dysentery and had been known as a natural drug for fungal infections. The leaf of *Acronychia pedunculata* is also commonly used to cure rheumatism, skin infection and stomach-ache (Perry, 1980).

Citrus is commonly well known as a very important genus belong to Rutaceae family. Citrus fruit also has a high economic value in food, beverage, flavour and pharmaceutical industry. *Citrus aurantifolia*, well known as a lime, has been used as a source of traditional medicine particularly for treatment of common cold and cough. In Malaysia Peninsular, the leaves of *C. aurantifolia* are commonly applied to relieve headache (Burkill and Haniff, 1986) and a decoction of the roots alleviates dysentery (Ridley, 1976). The fruit juice mixed with pulped *Phyllanthus spp.* is recommended for gonorrhea treatment and the juice of leaves pounded with *Areca sp.*, could be taken to treat stomach-ache (Anonymous, 1975). Recently, Guthrie *et al.* (1996) reported that juice of citrus fruit showing a strong inhibition activity on human breast cancer proliferation and delay the mammary tumorigenesis development.

In relation to the study and production of plant secondary metabolites, application of plant tissue culture technique offers many advantages compared to the intact plant (Butchner, 1977) such as :

(a) Relatively easy to grow and can be maintained under strictly controlled nutritional and environmental conditions.

(b) The uncertainties of climate and soils can be avoided.

(c) The problems associated with contamination by microorganisms can be avoided.

(d) Simple and more convenient in experimental system than intact plant.

(e) Very effective way of incorporating precursor materials which are often difficult to administer to the entire plant.

(f) Available for the relatively large scale production of plant cell-suspensions in batch cultures, chemostats and turbidostats.

The major application of plant tissue culture technique in citrus was in plant micropropagation. Previous research on the production of plant secondary metabolites from citrus tissue culture was very limited. All commercially valuable secondary products such as essential oils, flavones, hesperidin and pectin were generally obtained from extraction of fresh tissues (Brunet and Ibrahim, 1973). Recently Rio *et al.* (1991) reported the accumulation of neokatone and valence in the callus cultures of three citrus

species (*C. paradisi, C. limonia and C. aurantium*) in the level similar to those found in the exocarp of the mature fruit. Following that, Rio *et al.* (1992) also reported that the bioproduction flavonoids system of neohesperidin and naringin in the callus cultures of *Citrus aurantium* were also occurred.

The application of plant tissue cultures has also some limitations, mainly because many cultures do not produce significant amounts of the characteristic compounds of the plant from which they were derived. Many researches were done in order to develop strategies for overcoming the problem and some promising results have been obtained with elicitation methods. Many reports shown that addition of biotic elicitors were effective in inducing some plant secondary metabolites. For instance, the addition of yeast extract to the culture medium of *Thalictrum rugosum* could significantly enhance the accumulation of berberine in the culture. A transient increase of rosmarinic acid (2.5 fold) in cultured cells of *Lithospermum erythrorhizon* was also observed after addition of yeast extract to the cell-suspension culture medium (Mizukami *et al.*, 1992). In another study, the increasing level of phytoalexin aglycones pterocarpans , medicarpin and maakiain were observed in *Cicer arientinum* cell culture upon application of yeast extract elicitor (Weideman *et al.*, 1991).

Objectives of the study

The main objectives of the present study were :

1. To initiate and establish the callus tissue culture of *Citrus aurantifolia.*
2. To examine the callus growth and analyze soluble polyphenols content and flavonoids production (quercetin and hesperetin) from *C. aurantifolia* callus cultures.
3. To investigate the effect of yeast extract (elicitor) on callus growth, soluble polyphenols content and flavonoids production (quercetin and hesperetin) from *C. aurantifolia* tissue cultures.

CHAPTER TWO

LITERATURE REVIEW

Brief History of *Citrus aurantifolia* (Christm & Panzer) Swingle

Citrus aurantifolia synonyms to *Limonia aurantifolia*, *Citrus javanica* and *Citrus notissima*, has the Malaysian vernacular names as 'limau nipis' or "limau asam". There are different assumptions around the origins of *C. aurantifolia.* This citrus was believed to have originated in the east Indian archipelago. They were probably brought across the sea of Oman by Arabian sailors and transported to Egypt and Europe (Davies and Albrigo, 1994). On the other hand, the plant was believed to have originated in Malaysian particularly in northern Malaysia Peninsular (Ziegler and Wolfe, 1975).

Citrus is grown primarily between the latitudes of 40°N to 40°S. More northern and southern locations of commercial productions exist where temperatures are moderately influenced by oceans winds (Davis and Albigo, 1994). All genera of Citrus have certain characters in common as follows (Ziegler and Wolfe, 1975) :

(a) The plants are thorny shrubs or trees with fragrant white flower

(b) The leaves are compound in nature, have three leaflets, but reduced to the single terminal leaflets. While appearing to be simple leaves at first glance, their compound origin is shown by the joint where the blade attaches to the petiole.

(c) The petiole is often bordered lengthwise by blade-like extensions called wings. The presence or absence of the wings and its physical characteristics such as size and shape are useful characters in identifying species.

(d) Mature fruits have green, yellow, orange or red color. Peel of fruit possess abundant oil glands. The inner portion of the peel is a whitish, spongy materials known as albedo, while the outer is a colored portions containing oil glands and color bodies known as flavedo.

(e) The interior of the fruit, is divided into several segments and packed full of juice.

C. aurantifolia is a small, thin skinned and very acidic fruit with high content of citric acid (7-8%) and volatile oils (Simpson and Ogorzaly, 1986). The mature fruit of *C. aurantifolia* usually used as addition on food, beverage and for long time well known as traditional cosmetic and medicine. As a source of traditional medicine, *C. aurantifolia* has been used to treat several illness such as common cold and cough, rheumatism, prolaptus recti, high blood pressure, fever and used as expectorant (Perry,

1980 ; Heyne, 1987 and Anonymous, 1995). This citrus also shows having antibacterial and antifungal activity (Anonymous, 1975).

Citrus is a rich source of flavonoids. The high content of flavonoids is commonly concentrated in the leaf and skin peel (Davies, 1997). Much successful work had been done on citrus flavonoids especially to study the function and effect of this compounds to animal and human body system. It was reported that most of citrus flavonoids possess an important function in mammalian enzyme systems. Some of flavonoids showed anti-inflammatory, anti-bacterial, anti-fungal, anti-tumour, and anti-viral activity in animals or in cell culture. Certain flavonoids from citrus species i.e. rutoside, triethylrutoside, and quercitroside also showed strong choleretic activity *in vitro* (Anonymous, 1975 ; Middleton, 1994 and Musci, 1985).

The main flavonoids occurred in citrus are flavanones, flavones, and anthocyanins. Another citrus flavonoids also found in a small content are aurone, leucoanthocyanins, catechins, isoflavones and dihydochalcones. The flavonoids are distributed throughout all the tissues of citrus fruit. The citrus flavanones (naringenin, isosakuranetin, eriodictyol and hesperetin) do not occur as the free aglycones but are combined through the C-7 hydroxy group with either β-rutinose (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) or β-neo-hesperidose (2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose). The flavanone neohesperidosides are distinguished from the flavanone

rutinosides by its taste, the neohesperidosides are bitter and the rutinosides are tasteless. A number of the common flavones (apigenin, acacetin, luteolin) are found as glycosides and it usually β-rutinose and β-hesperidose linked through the C-7 hydroxy of the flavone (Kefford and Chaudler, 1970 and Nagy *et al.*, 1977).

Hesperidin (hesperetin 7-rutinoside), the most common flavanone which is present in all commercial species of citrus was found as major flavanone in *C. aurantifolia*. This compound is capable of preventing abnormal capillary permeability and together with naringin, nobiletin and tangeretin were proved acting as anti-bacterial mutagenesis compounds. Naringin and hesperidin also posses a weak antimutagenic activity against benzo(*a*)pyrene (BaP) and nobiletin act as anti-mutagen against 2-aminofluerene (Calomme *et al.*, 1996). Naringenin, eriodictyol and luteolin were reported having important functions as vasodilatory active flavonoids which could reduce the risk of coronary heart incindents (Sanchez *et al.*, 1995).

Hesperidin widely distributed in various tissues of *C. aurantifolia* whereas the highest concentration (3.3 $mg.g^{-1}$.ft.wt) is located in the peel of fruit and usually occurred in association with vitamin C (ascorbic acid). Some symptoms originally thought to be due to vitamin C deficiency such as bruising due to capillary fragility were found in early studies to be relieved by

crude extracts of vitamin C but not by purified vitamin C and hesperidin were found to be the essential component in correcting this bruising tendency and improving the permeability and integrity of the capillarity lining. Hesperidin deficiency has been linked with abnormal capillary leakness as well as pain in the extremities causing itchiness, weakness and night leg cramps. Supplemented hesperidin may also help to reduce edema or excess swelling in the legs due to fluid accumulation (Davies, 1997).

Hesperetin, an aglycone of hesperidin is a naturally occurring flavonoid. It is interesting because of their anti-cancer, anti-oxidant and anti-viral properties which can be useful in fighting many diseases Middleton (1994) reported that hesperetin has an ability to reduce the intracellular replication of HSV-1, polivirus type 1, parainfluenza virus type 3 and syncytial virus (RSV). Hesperetin was also reported having a positive effect in increasing ocular blood flow. This finding indicated that this compound could be used to treat ischemic eye disease in the future (Liu *et al.*, 1996).

Rutin is also found in high concentration particularly in leaf and peel of *Citrus* fruit. Rutin is a non-mutagenic flavonol glycoside, whereas its aglycone, quercetin is mutagenic. Rutin partially protected oxy-Hb against H_2O_2-induced oxidation and heme loss. Rutin was also shown to delay H_2O_2-induced meta-Hb oxidation to ferryl-Hb and directly reduced ferryl-Hb to met-