UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND ANTIBACTERIAL PROPERTIES OF FLAVOUR AND FLAVONOID COMPOUNDS FROM CULTURED TISSUES OF *CITRUS HYSTRIX* D.C. ('LIMAU PURUT').

SURI BIN ROOWI

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By

SURI BIN ROOWI

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

May 2001

DEDICATION

"Dedicated especially to mom (Jainab), my late father (Hj Roowi), my wife (Zaharinah), my sons (Abu Hanifa and Zaid), my brothers and sisters (Hj. Sumali, Kalsom, Basirah, Dayah, Ahmad and family, Habsah, Jumeyah, Amnah), my father and mother-in-law and those whose sacrifice and support me to complete my study successfully."

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

PRODUCTION AND ANTIBACTERIAL PROPERTIES OF FLAVOUR AND FLAVONOID COMPOUNDS FROM CULTURED TISSUES OF *CITRUS HYSTRIX* D.C. (`LIMAU PURUT')

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May 2001

Chairman : Associate Professor Radzali Muse, Ph.D.

Faculty : Science and Environmental Studies

Analyses of the flavour compounds from the selected parts of flower, seedlings grown *in-vitro* and callus cultures of *Citrus hystrix* D.C. were performed using the gas chromatography (GC) technique. The results showed that the major flavour compound obtained from the flower was citronellal. Young seedlings grown *in-vitro* on the Murashige and Skoog (MS) basal medium without plant growth regulators did not produce any citronellal. Nevertheless, the quantity of limonene was remarkably higher (101.00 $\pm$ 5.24 μg/g fwt. tissue) than in the petal and ovary and pollen plus anther. Callus derived from stem treated with 2.0 mg/l (w/v) NAA plus 1.0 mg/l (w/v) kinetin reached a maximum growth (0.94 $\pm$ 0.08 g fwt./culture) after six weeks of culture. No callus was found from the leaf and peel. Maximum production of flavour compounds i.e. cyclohexanol, limonene and β-pinene were obtained after three and four weeks of culture.

Treatment of the *C. hystrix* stem-derived callus with 1.0 mg/l (w/v) of kinetin showed higher growth (0.42 ± 0.04 g fwt./culture) than all treatment with NAA. Most of the flavour compounds in the callus were found highest after being treated with 5.0 mg/l (w/v) of NAA. Addition of various concentrations of salicylic acid (0 to 20.0 mM), yeast extract (0 to 0.5%) and alginate (0 to 0.5%) into the medium, decreased the callus growth. However, treatment of stem-derived callus with 0.3% of (w/v) of alginate resulted in higher growth (0.88 ± 0.05 g fwt./culture) than the other treatments. On the other hand, callus treated with yeast extract and salicylic acid were able to syntheses two and six additional compounds respectively compared to the control. Treatment of *C. hystrix* stem-derived callus with proline and phenylalanine decreased the callus growth but significantly increased the production of flavour compounds i.e. p-cymene, terpineol, citronellal, citronellol, γ-terpene and β-pinene.

Analyses of flavonoid compounds from young seedlings, callus and different parts of *C. hystrix* intact plant such as leaf, flower, stem and fruit was performed by high performance liquid chromatography technique (HPLC). The highest production of flavonoids i.e. naringin (11.66 ± 0.76 mg/g dwt. tissue), rutin (34.63 ± 1.69 mg/g dwt. tissue) and kaempferol (3.01 ± 0.02 mg/g dwt. tissue) was found in the peel; hesperidin (3.21 ± 0.23 mg/g dwt. tissue) in the leaf and quercetin (0.68 ± 0.04 mg/g dwt. tissue) in the whole flower. In the young seedlings, naringin (5.26 ± 0.25

mg/g dwt. tissue), and rutin (0.91 ± 0.03 mg/g dwt. tissue) were found in high concentration in the stem compared to the leaf and root. The naringin and rutin content of stem-derived callus showed the maximum values at 2.29 ± 0.09 and 0.90 ± 0.03 mg/g dwt. tissue respectively after six weeks of culture.

Treatment of stem-derived callus with 0.3% and 0.5% (w/v) of agarose gave the highest production of naringin and rutin at 12.13 ± 0.07 and 3.09 ± 0.05 mg/g dwt. tissue, respectively. Addition of 2.0 mM phenylalanine into the culture medium also increased the production of naringin (24.05 ± 1.02 mg/g dwt. tissue) and rutin (3.52 ± 0.12 mg/g dwt. tissue).

The essential oils (contains flavour compounds) and the methanolic extracts (contains flavonoid compounds) were obtained from peel, leaf, juice and stem-derived callus. Results showed that most extracts were able to inhibit gram positive and gram negative bacterial growth.

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

PENGHASILAN DAN SIFAT ANTIBAKTERIA PADA BAHAN PERISA DAN FLAVONOID DARI KULTUR TISU LIMAU PURUT (*CITRUS HYSTRIX* D.C.)

Oleh

SURI BIN ROOWI

Mei 2001

Pengerusi : Profesor Madya Radzali Muse, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Analisis kandungan bahan perisa pada beberapa bahagian bunga limau purut (*Citrus hystrix*), anak pokok yang ditanam secara *in-vitro* dan kalus telah dilakukan menggunakan teknik kromatografi gas (GC). Hasil ujikaji menunjukkan komponen bahan perisa yang tertinggi adalah sitronelal. Anak pokok yang ditumbuhkan di atas media asas pertumbuhan Murashige dan Skoog (MS) tanpa pengawal atur pertumbuhan tidak berkemampuan mensintesis bahan sitronelal. Walau bagaimanapun, kandungan bahan limonen didapati lebih tinggi (101.00 $\pm$ 5.24 μg/g berat basah tisu) berbanding dengan kelopak, ovari dan debunga dan cepu debunga (anter). Kalus yang diaruh dari batang menggunakan 2.0 mg/l (berat/isipadu) asid α-naftalena asetik (NAA) dan 1.0 mg/l (berat/isipadu) kinetin menunjukkan pertumbuhan yang tertinggi (0.94 $\pm$ 0.10 g berat basah/kultur) selepas 3 dan 4 minggu pengeraman. Sebaliknya, tiada pembentukan kalus pada

bahagian lain seperti daun dan kulit. Penghasilan bahan perisa maksima contohnya sikloheksanol, limonena dan β-pinena seterusnya didapati selepas tiga dan empat minggu pengkulturan.

Kalus batang yang telah dirawat menggunakan 1.0 mg/l (berat/isipadu) kinetin menunjukkan pertumbuhan yang lebih tinggi (0.42 ± 0.04 g berat basah/kultur) berbanding dengan rawatan menggunakan NAA. Kebanyakkan kuantiti bahan perisa tertinggi didapati pada kalus yang dirawat dengan 5.0 mg/l (berat/isipadu) NAA. Rawatan menggunakan asid salisilik, ekstrak yis dan alginat pada kepekatan yang berbeza, telah merencatkan pertumbuhan kalus. Sebaliknya, rawatan kultur kalus dengan menggunakan 0.3% (berat/isipadu) alginat pula telah mencatatkan pertumbuhan kalus yang lebih tinggi (0.88 ± 0.05 g berat basah/kultur). Hasil analisis kandungan perisa menunjukkan bahawa rawatan dengan menggunakan ekstrak yis dan asid salisilik berkeupayaan mensintesis dua dan enam komponen perisa berbanding dengan kawalan. Penambahan bahan prolina dan fenilalanina ke dalam media pertumbuhan kalus *C. hystrix* juga telah merencatkan pertumbuhan kalus tetapi meningkatkan penghasilan bahan perisa contohnya p-simena, terpineol, sitronellal, sitronellol, γ-terpinena dan β-pinena.

Analisis sebatian flavonoid daripada anak pokok, kalus dan bahagian pokok yang berbeza contohnya daun, bunga, batang dan buah pada

C. hystrix telah dilakukan dengan menggunakan teknik kromatografi turus berprestasi tinggi (HPLC). Tahap penghasilan bahan flavonoid tertinggi contohnya naringin (11.66 ± 0.76 mg/g berat kering tisu), rutin (34.63 ± 1.69 mg/g berat kering tisu) dan kaempferol (3.01 ± 0.02 mg/g berat kering tisu) di dapati di dalam kulit buah; hesperidin (3.21 ± 0.23 mg/g berat kering tisu) dalam daun dan kuercetin (0.68 ± 0.04 mg/g berat kering tisu) dalam bunga. Naringin (5.26 ± 0.25 mg/g berat kering tisu) dan rutin (0.91 ± 0.03 mg/g berat kering tisu) juga didapati tinggi pada batang anak pokok. Kalus *C. hystrix* juga mencatatkan penghasilan naringin (2.29 ± 0.09 mg/g berat kering tisu) dan rutin (0.90 ± 0.03 mg/g berat kering tisu) maksimum selepas enam minggu pengkulturan.

Rawatan kalus batang dengan menggunakan 0.3 dan 0.5% (berat/isipadu) agarosa telah menghasilkan naringin (12.13 ± 0.07 mg/g berat kering tisu) dan rutin (3.09 ± 0.05 mg/g berat kering tisu) tertinggi. Penambahan 2.0 mM fenilalanina ke dalam kultur media juga boleh meningkatkan penghasilan naringin (24.05 ± 1.02 mg/g berat kering tisu) dan rutin (3.52 ± 0.12 mg/g berat kering tisu).

Kajian ke atas perencatan pertumbuhan bakteria dengan menggunakan minyak pati yang mengandungi bahan perisa dan ekstrak metanol yang mengandungi bahan flavonoid, juga telah dilakukan. Hasil kajian telah menunjukkan bahawa hasil ekstrak berkemampuan merencatkan pertumbuhan bakteria gram positif dan negatif.

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I also wish to thank my fellow lab-mates for their encouragement and support and last but not least, my special thank goes to my parent for their endless love.

I certify that an Examination Committee met on 9th May 2001 to conduct the final examination of Suri Bin Roowi on his Master of Science thesis entitled "Production and Antibacterial Properties of Flavour and Flavonoid Compounds from Cultured Tissues of *Citrus hystrix* D.C. (`Limau Purut')" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Johari Ramli, Ph.D
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Date: 23 MAY 2001

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

AINI IDERIS, Ph.D,
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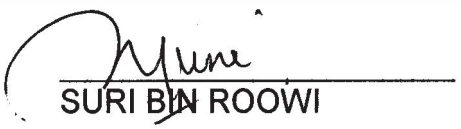
Date: 14 JUN 2001

DECLARATION

I hereby declare that the thesis is based on my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



SURI BIN ROOWI

Date: 23 MAY 2001

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LIST OF ABBREVIATIONS

Abs	- absorbance
CH	- casein hydrolysate
cm	- centimeter
dwt.	- dry weight basis
FID	- flame ionized detector
fwt.	- fresh weight
g	- gram
GC	- gas chromatography
HP	- Hewlett Packard
HPLC	- high performance liquid chromatography
i.d.	- inner diameter
kg	- kilogram
m	- meter
M	- molar
M	- magnificent
µg	- microgram
µl	- microliter
µM	- micromolar
mg	- milligram
min	- minute
ml	- milliliter
MS	- Murashige and Skoog
N	- normality
NAA	- α-naphthalene acetic acid
NaDEDTC	- sodium diethyldithiocarbamate
PAL	- phenylalanine ammonium lyase
PEG	- polyethylene glycol
PGRs	- plant growth regulators
PITC	- phenylisothiocynate
RI	- retention index
SA	- salicylic acid
SDA	- simultaneous distillation adsorption
SDE	- simultaneous distillation extraction
TEA	- triethylamine
TFA	- trifluoroacetic acid
Tiss.	- tissue
U	- unit
YE	- yeast extract
(w/v)	- weight/volume
(w/w)	- weight/weight

CHAPTER I

INTRODUCTION

Secondary metabolites can be defined as compounds which are biosynthetically derived from primary metabolites (Blandrin and Klocke, 1985). These include alkaloids, phenolic compounds, flavonoids, steroids, carotenoids and terpenoids. Among the most valuable plant secondary metabolites are vinblastine (anticancer) , vincristine (antileukemia) and taxol (anticancer), scopolamine (sedative drug), atropine (as anticholergen) and digoxin (heart stimulants) (Collin and Edwards, 1998). In the higher plants, these compounds are frequently produced in small quantities compared to the primary metabolites and need special cell types at distinct developmental stages (Blandrin and Klocke, 1985).

Secondary metabolites are compounds which have no apparent function in primary metabolism but often have ecological roles, e.g. as a pollinator, represent chemical adaptations to environmental stress, or serve as chemical defenses against microorganism, insects and higher predators (Harborne, 1982). Secondary metabolites were also used to detoxify substances accumulated during primary metabolism, act as physiological effectors, provisioned of chemical signals to coordinate the metabolism of cells and used to develop the ecological relationship (Luckner, 1986). However, due to the difference in structure and their sites of location in

plants, it is difficult to identify a common function of all secondary metabolites (Collin and Edwards, 1998). Some secondary metabolites may have detrimental effects on man and domesticated animals. For example, an excessive intake of certain secondary metabolites can inhibit digestive enzymes, induce Parkinson and Alzheimer disease, anemia, toxic and liver damage (Endress, 1994).

Most spices, condiments, tea, citrus and other plants produce their secondary metabolites for specific properties, for example flavour and fragrance. Although such compounds can be produced synthetically, high prices are still being paid in some cases for extracted compounds from the natural sources, especially to be used as food additives, natural colour or flavouring agents (Blandrin and Klocke, 1985). For example, synthetic benzaldehyde sells at US$1.50 to US$1.75/kg whereas natural benzaldehyde sells for over US$200/kg. Similarly, synthetic methyl anthranilate sells for US$5.75/kg compared to natural methyl anthranilate which sells at more than US$400/kg to US$500/kg (Sahai, 1994). The market for natural flavours in the US was estimated at US$200 million per year and there have been an increasing demand on the use of natural flavours rather than synthetic flavours which were obtained through organic synthesis (McCormick and Wild, 1989).

Citrus flavour is widely used to flavour or aromatize food, non-carbonated or carbonated beverages and also in household products as it