UNIVERSITI PUTRA MALAYSIA

ANTI-TUMOUR PROMOTING ACTIVITY OF SELECTED MALAYSIAN VEGETABLES AND FRUITS, AND IDENTIFICATION OF ANTI-TUMOUR PROMOTING AND ANTIOXIDANT COMPOUNDS FROM *COLEUS TUBEROSUS*, BENTH (UBI KEMILI)

LIM YANG MOOI

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By
LIM YANG MOOI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

May 2002

Specially dedicated to my beloved

Grandmother,

Father, Mother,

Brothers, Sisters,

Husband,

Parents-in-law,

And friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Doctor of Philosophy

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May 2002

Chairman : Professor Dr. Abdul Manaf Ali,

Faculty : Faculty of Food Science and Biotechnology

A convenient short-term *in vitro* assay, the inhibition of Epstein-Barr virus (EBV) activation induced by phorbol 12-myristate 13-acetate (PMA) and sodium *n*-butyrate was conducted to detect the naturally occurring anti-tumour promoters of 133 vegetables and fruits. Forty-two plants showed strong inhibitory activity, 33 plants showed moderate inhibitory activity, 21 plants were found to be weakly active and 37 plants were inactive. *Coleus tuberosus*, Benth was chosen for further study because it showed the strongest activity. Phytosterols (CT 1) and 2α,3β-dihydroxyl-12-oleanen-28-oic-acid (CT 2) were isolated from *Coleus tuberosus*, Benth based on bioassay-guided fractionation. CT 1 was identified as a mixture consisting of stigmasterol (32.0%), campesterol (27.7%) and β-sitosterol (40.3%) by gas chromatography. CT 2 was established as 2α,3β-dihydroxy-12-oleanen-28-oic acid. Five plants that showed the highest anti-tumour promoting activity namely, *Carica papaya*, *Barringtonia macrostachya*, *Coleus tuberosus*, *Mangifera indica* and *Eugenia polyantha* also exhibited strong antioxidant activity compared to α-tocopherol in the ferric thiocyanate (FTC) method and showed more than 60%

inhibition rate in the xanthine/xanthine oxidase system. Those plants mentioned above did not exhibit any activity in scavenging stable DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals and hydrogen peroxide stimulated in differentiated HL 60 cells by PMA. Isolated compounds CT 1 and CT 2 and commercial standards campesterol, stigmasterol, and β-sitosterol were also tested for their antioxidant activity. Campesterol, CT 1, stigmasterol, and β-sitosterol demonstrated more than 50% inhibition rate in scavenging superoxide anion induced in the xanthine/xanthine oxidase system. CT 2 showed an inhibition rate of 46.62%. Campesterol, CT 1 and β-sitosterol showed more than 50% inhibition rate at 2 μg/ml in scavenging hydrogen peroxide induced in differentiated HL 60 cells, but CT 2 and stigmasterol, attained an inhibition rate of only 32.97% and 16.37%, respectively. All compounds did not exhibit any activity in scavenging stable DPPH radicals. Campesterol, CT 1, CT 2, stigmasterol and β-sitosterol were found to have very strong anti-tumour promoting activity and their IC_{50} were determined as 2 μg/ml, 0.7 μg/ml, 0.1 μg/ml, 0.6 μg/ml and 1 μg/ml, respectively. The optimum combination effect of stigmasterol, β-sitosterol and campesterol towards the anti-tumour promoting activity was obtained at 40 μg/ml of each compound analysed by the response surface methodology (RSM). Campesterol was assessed to cause a negative interaction to β-sitosterol, while, stigmasterol caused a negative interaction to campesterol at concentrations higher than 20 μg/ml. Immunoblotting analysis was used as a confirmation test for the detection of the EBV early antigen EA-D and EA-R. EA-D was detected as a darker band of about 50-52 kDa while EA-R showed up as a fade band of about 85 kDa. In conclusion, CT 1 and CT 2 were found to show strong anti-tumour promoting activity. This supports the assumption that the consumption

of vegetables and fruits is a highly recommended strategy for cancer chemoprevention and can be a practical approach to control cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
untuk memenuhi keperluan untuk ijazah Doktor Falsafah

AKTIVITI ANTI-PENGGALAK TUMOR BAGI SAYUR-SAYURAN DAN BUAH-BUAHAN MALAYSIA TERPILIH DAN PENGENALPASTIAN SEBATIAN ANTI-PENGGALAK TUMOR DAN ANTIOXIDANT DARIPADA *COLEUS TUBEROSUS*, BENTH (UBI KEMILI)

Oleh

LIM YANG MOOI

Mei 2002

Pengerusi: **Profesor Dr. Abdul Manaf Ali,**

Fakulti: **Fakulti Sains Makanan dan Bioteknologi**

Suatu bio-cerakin *in vitro* jangka pendek telah dijalankan iaitu perencatan pengaktifan virus Epstein-Barr (EBV) yang diaruh oleh forbol 12-miristat 13-asetat (PMA, 5 μM) dan 3 mM natrium *n*-butirat untuk mengesan anti-penggalak tumor semulajadi dalam 133 sayur-sayuran and buah-buahan. Empat puluh dua tumbuhan telah menunjukkan aktiviti perencatan yang tinggi, 33 tumbuhan menunjukkan aktiviti perencatan sederhana, 21 menunjukkan aktiviti perencatan lemah dan 37 tumbuhan pula tidak aktif. *Coleus tuberosus*, Benth telah dipilih untuk kajian selanjutnya kerana menunjukkan aktiviti perencatan yang tertinggi. Dua sebatian iaitu satu campuran fitosterol (CT 1) dan asid 2α,3β-dihidroksida-12-oleanen 28-oik (CT 2) telah diperolehi daripada pokok *Coleus tuberosus*, Benth dengan mengaplikasikan teknik pengasingan berpandukan biocerakinan. CT 1 telah dikenalpasti sebagai campuran sebatian yang terdiri daripada stigmasterol (32.0%), kampesterol (27.7%) dan β-sitosterol (40.3%) dengan kaedah kromatografi gas. CT 2 pula telah dikenalpasti sebagai asid 2α,3β-dihidroksida-12-oleanen 28-oik. Lima tumbuhan terdiri daripada *Carica papaya*, *Barringtonia macrostachya*, *Coleus*

tuberosus, Mangifera indica dan *Eugenia polyantha* yang menunjukkan perencatan pengaktifan Epstein-Barr virus yang tinggi juga menunjukkan aktiviti antioxidan yang tinggi berbanding dengan α-tokoferol melalui kaedah Ferik tiosianat (FTC), dan menunjukkan lebih daripada 60 % kadar perencatan dalam sistem xanthina / xanthina oksidase. Lima tumbuhan tersebut tidak memberi sabarang perencatan terhadap aktiviti menghilangkan radikal 1,1-difenil-2-pikrilhidrazil (DPPH) yang stabil dan hidrogen peroksida yang dihasilkan dalam sel-sel HL 60 yang telah distimulasikan untuk melalui proses perbezaan oleh PMA. Ujian aktiviti antioxidant pada sebatian CT 1 and CT2 dan komersial piawai iaitu stigmasterol, kampesterol dan β-sitosterol juga dijalankan. CT 1, stigmasterol, kampesterol dan β-sitosterol telah terbukti menunjukkan kadar perencatan melebihi 50% dalam sistem xanthina / xanthina oksidase. CT 2 menunjukkan kadar perencatan pada 46.62%. CT 1, kampesterol dan β-sitosterol merencat penghasilan hidrogen peroksida dalam sel-sel HL 60 yang telah melalui pembezaan pada kadar perencatan melebihi 50% pada kepekatan 2 μg/ml. CT 2 dan stigmasterol masing masing menunjukkan 32.97% dan 16.37% kadar perencatan pada kepekatan 2 μg/ml. Sebatian ini juga tidak menunjukkan sebarang aktiviti menghilangkan radikal DPPH yang stabil. CT 1, CT 2, stigmasterol, kampesterol dan β-sitosterol telah menunjukkan aktiviti anti-penggalak tumor yang tinggi dan memberi IC_{50} pada 0.7 μg/ml (CT 1), 0.6 μg/ml (stigmasterol), 1 μg/ml (β-sitosterol), 2 μg/ml (kampesterol) dan 0.1 μg/ml (CT 2). Kesan kombinasi optimum stigmasterol, kampesterol dan β-sitosterol bagi aktiviti promoter tumor telah dicapai pada 40 μg/ml bagi setiap sebatian yang dianalisiskan melalui kaedah gerakbalas permukaan (RSM). Kampesterol telah ditaksir untuk menyebabkan interaksi negatif kepada β-sitosterol, sementara, stigmasterol pula menyebabkan interaksi negatif kepada kampesterol pada konsentrasi yang lebih

tinggi daripada 20 μg/ml. Analisis imunokedap dengan menjalankan Western blotting dapat digunakan sebagai ujian pemastian bagi pengesanan antigen EA-D dan EA-R. Antigen EA-D dan EA-R telah dikesan melalui kaedah imunokedap sebagai jalur hitam pada kira-kira 50-52 kDa dan jalur kabur pada kira-kira 85 kDa. Pada kesimpulannya, CT 1 and CT 2 menunjukkan aktiviti promoter anti-tumor yang tinggi. Ini menyokong anggapan iaitu memakan sayur-sayuran and buah-buahan adalah satu strategi yang amat disyorkan bagi rawatan kanser secara kimo-pencegahan dan merupakan pendekatan yang praktikal untuk mengawal kanser.

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LIST OF ABBREVIATIONS

ACF	:	Aberrant crypt foci
°C	:	Degree Celsius
CGM	:	Complete growth medium
4CN	:	4-Chloro-1-Naphthol
CO_2	:	Carbon dioxide
COSY	:	Correlated spectroscopy
DCF	:	Dichlorofluorescein
DCFH	:	Dichlorofluorescin
DCFH-DA	:	2',7'-dichlorofluorescin diacetate
DEPT	:	Distortionless enhancement by polarisation transfer
DMFO	:	2-difluoromethylornithine
DMSO	:	Dimethyl sulfoxide
DPPH	:	1,1-diphenyl-2-picrylhydrazyl
DTT	:	Dithiothreitol
EA	:	Early antigen
EMIS	:	Electron impact mass spectrometry
FCS	:	Foetal calf serum
FITC	:	Flouresceinthiocynate
FT-IR	:	Fourier transform mass spectroscopy
GST	:	Gluthathione-*S*-transferase
HCl	:	Hydrochloric acid
HL-60	:	Human promyelocytic cell
HMBC	:	Heteronuclear multiple bond correlation
HMQC	:	Heteronulear multiple quantum correlation
Hp	:	Horseradish Peroxidase
IR	:	Infrared
J	:	Coupling constant
m/z	:	Mass to charge ratio
M	:	Molar
MDA	:	Malon-dialdehyde
mg	:	Milligram
MS	:	Mass spectrum
ml	:	Millilitre
min	:	Minutes
mA	:	Milliampere
NADPH	:	Reduced nicotinamide adenine dinucleotide phosphate
NBT	:	Nitroblue tetrazolium
nm	:	Nanometer
NMR	:	Nuclear magnetic resonance
NPC	:	Nasopharyngeal carcinoma
NSAID	:	Nonsteroidal anti-inflammatory drug
no	:	Number
ODC	:	Ornithine decarboxylase
PBS	:	Phosphate buffered saline
PKC	:	Protein Kinase C
PMA	:	Phorbol 12-myristate 13-acetate
PMSF	:	Phenylmethylsulfonyl fluoride
QR	:	Quinone reductase

ROS	:	Reactive oxygen species
RSM	:	Response Surface Methodology
RPMI	:	Rosewell Park Memorial Institute
rpm	:	Rotation per minute
SDS	:	Sodium dodecyl sulphate
SOD	:	Superoxide dismutase
TEMED	:	*N,N,N',N'*-tetramethylenediamine
Tris	:	Tris (hydrosymethl) aminoethane
TMS	:	Tetramethylsilane
TPA	:	12-*O*-tetradecanol-phorbol-13-acetate
UV	:	Ultraviolet
XOD	:	Xanthine Oxidase
μ	:	Micro
%	:	Percentage
δ	:	Chemical shift
λ_{max}	:	In UV spectroscopy, the wavelength at which maximum absorption occurs