CYTOTOXIC AND APOPTOTIC ACTIVITIES IN SELECTED PHYLLANTHACEAE SPECIES OF MALAYSIA

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Field of Study : Bioactivity of Natural Product

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ABSTRACT

Phyllanthaceae species have been extensively used in folk medicine in most tropical and subtropical countries for thousand of years. However, there is a paucity of information on the cytotoxic properties of these plants. Therefore, the present study was undertaken to evaluate the cytotoxic and apoptotic activity of crude methanol (CME), hexane (CHE) and ethyl acetate (CEE) extracts of the selected Phyllanthaceae species collected from different parts of Peninsular Malaysia, namely *Phyllanthus niruri* (dukong anak), *P. pectinatus* (Pokok Melaka), *P. acidus* (cermai), *P. roseus* (Labu Kuncing), *P. watsonii* and *Baccaurea motleyana* (rambai). Cytotoxic activities were screened using an *in vitro* assay system of growth inhibition against four human cancer cell lines, namely breast cancer cells (MCF7), ovarian cancer cells (SKOV3), epidermal carcinoma of cervix cells (CaSki), colon cancer cells (HT29), and one normal lung fibroblast cells (MRC5). CME and CEE of *P. pectinatus* (leaves) exhibited potent cytotoxic activity against SKOV3 cells with IC_{50} values of 4.8 ± 1.04 and 5.8 ± 0.76 μg/ml, respectively. CEE of *P. pectinatus* (fruit) exhibited strong cytotoxicity on MCF7 and CaSki cells with IC_{50} values of 18.1 ± 0.66 and 19.4 ± 0.53 μg/ml, respectively. CHE of *P. watsonii* exhibited strong cytotoxicity with IC_{50} values of 7.9 ± 0.60, 5.8 ± 0.29, 6.9 ± 0.96 and 11.8 ± 1.61 μg/ml on MCF7, SKOV3, CaSki and HT29 cells, respectively. CEE of *P. watsonii* demonstrated the potent cytotoxicity with an IC_{50} value of 3.6 ± 1.01 μg/ml on CaSki cells, as well as on HT29 and SKOV3 cells with IC_{50} values of 5.1 ± 0.36 and 5.5 ± 0.50 μg/ml, respectively. CHE of *P. watsonii* was further subjected for bioassay-guided fractionation and yielded 10 fractions (PW1 – PW10). PW4 – PW8 portraying a stronger cytotoxic activity against MCF7, SKOV3, CaSki and HT29 cells and was further subjected for bioassay-guided fractionation and this

resulted in 8 fractions (PPW1 – PPW8). Cytotoxic activity of fraction PPW7 on MCF7, SKOV3, CaSki and HT29 cells were more active with IC_{50} values of 0.9 ± 0.06, 0.7 ± 0.06, 0.8 ± 0.00 and 0.8 ± 0.10 μg/ml. Cytotoxic activity of CME, CHE and CEE of *P. watsonii* and fraction PPW7 were shown to be quite selective for cancer cells with selectivity index ranging from 4.4 to 14.6. Fraction PPW7 was subjected to LC-MS/MS analysis and six main compounds were identified. The compounds detected were ellagic acid, geranic acid, glochidone, betulin, phyllanthin and sterol glucoside. Marked morphological changes, ladder-like appearance of DNA and increment in caspase-3 activity indicating of apoptosis were clearly observed in MCF7, SKOV3, CaSki and HT29 cells-treated with cytotoxically active crude extracts of *P. pectinatus* (leaves and fruits), and *P. watsonii*, and fractions PPW6 and PPW7. It was also observed that CHE of *P. watsonii* arrested MCF7, SKOV3 and CaSki cells at G_0/G_1- S, S-G_2/M and G_0/G_1- G_2/M phases, and fraction PPW7 arrested SKOV3 cells at S and G_2/M phases. Cytotoxic activity of endemic *P. watsonii* collected directly from Endau-Rompin Park, Johor against MCF7, SKOV3, CaSki and HT29 cells were investigated for the first time. These results demonstrated that *P. watsonii* has strong cytotoxic effect by inducing apoptotic cell death, increasing caspase-3 activity, and causing arrest of cancer cells at different growth phases. Hence, *P. watsonii* has the potential to be further exploited for the discovery and development for new anticancer pharmaceuticals.

ABSTRAK

Spesies Phyllanthaceae telah lama digunakan secara intensif dalam perubatan tradisional di kebanyakan negara tropika dan subtropika selama beribu-ribu tahun. Walaubagaimanapun, maklumat mengenai nilai sitotoksik spesies Phyllanthaceae ini masih kurang. Oleh sebab itu, kajian ini telah dijalankan untuk menilai aktiviti sitotoksik and apoptotik ekstrak mentah methanol (CME), heksane (CHE) dan etil asetat (CEE) bagi spesies Phyllanthaceae daripada enam spesies Phyllanthaceae yang dikutip dari kawasan-kawasan berlainan di Semenanjung Malaysia iaitu, *Phyllanthus niruri* (dukong anak), *P. pectinatus* (Pokok Melaka), *P. acidus* (cermai), *P. roseus* (Labu Kuncing), *P. watsonii* dan *Baccaurea motleyana* (rambai). Penyaringan aktiviti sitotoksik telah dijalankan menggunakan sistem asei perencatan pertumbuhan secara *in vitro* ke atas empat leluhur sel karsinoma manusia, iaitu sel kanser payudara (MCF7), sel kanser ovari (SKOV3), sel karsinoma epidermal serviks (CaSki), sel kanser kolon (HT29), dan sel normal fibroblas paru-paru (MRC5). CME dan CEE bagi spesies *P. pectinatus* (daun) mempamerkan kesan sitotoksik yang kuat ke atas SKOV3 dengan nilai IC_{50} sebanyak 4.8 ± 1.04 dan 5.8 ± 0.76 μg/ml, masing-masing. CEE dari *P. pectinatus* (buah) mempamerkan aktiviti sitotoksik yang kuat ke atas sel MCF7 dan CaSki dengan nilai IC_{50} sebanyak 18.1 ± 0.66 dan 19.4 ± 0.53 μg/ml. CHE dari *P. watsonii* mempamerkan sitotoksiti yang kuat dengan nilai IC_{50} sebanyak 7.9 ± 0.60, 5.8 ± 0.29, 6.9 ± 0.96 dan 11.8 ± 1.61 μg/ml ke atas sel MCF7, SKOV3, CaSki dan HT29, masing-masing. CEE dari *P. watsonii* menunjukkan aktiviti sitotoksik yang paling baik dengan nilai IC_{50} sebanyak 3.6 ± 1.01 μg/ml ke atas sel CaSki, begitu juga ke atas sel HT29 dan SKOV3 dengan nilai IC_{50} sebanyak 5.1 ± 0.36 and 5.5 ± 0.50 μg/ml, masing-masing. CHE dari *P. watsonii* disubjekkan ke atas pencerakinan bioasei secara pemanduan

dan menghasilkan 10 fraksi (PW1 – PW10). PW4 – PW8 mempamerkan aktiviti sitoktoksik yang paling kuat ke atas sel MCF7, SKOV3, CaSki and HT29 disubjekkan ke atas pencerakinan bioasei secara pemanduan dengan lebih lanjut dan menghasilkan pencerakinan sebanyak 8 fraksi (PPW1 – PPW8). Aktiviti sitoksoksik fraksi PPW7 ke atas sel MCF7, SKOV3, CaSki dan HT29 adalah paling mujarab dengan nilai IC_{50} sebanyak 0.9 ± 0.06, 0.7 ± 0.06, 0.8 ± 0.00 dan 0.8 ± 0.10 μg/ml. Ciri sitoktoksik bagi CME, CHE dan CEE bagi *P. watsonii* dan fraksi PPW7 menunjukkan selektiviti ke atas kanser sel dengan indeks selektiviti berjulat dari 4.4 ke 14.6. Fraksi PPW7 disubjekkan ke atas analisa LC-MS/MS dan enam sebatian utama dapat dikenalpasti. Sebatian-sebatian yang dikesan adalah asid ellagic, asid geraiinic, glochidone, betulin, phyllanthin dan sterol glukosida. Perubahan ciri-ciri morfologi, DNA kelihatan seperti tangga dan peningkatan dalam aktiviti caspase-3 yang menandakan apoptosis sel dapat dilihat dengan jelas di dalam sel MCF7, SKOV3, CaSki dan HT29 yang dirawat dengan ekstrak mentah yang aktif dalam sitotoksik dari *P. pectinatus* (daun dan buah) dan fraksi PPW6 dan PPW7. Ia juga didapati bahawa CHE dari *P. watsonii* merencat sel MCF7, SKOV3 dan CaSki pada fasa G_0/G_1- S, S-G_2/M dan G_0/G_1- G_2/M, dan fraksi PPW7 merencat sel SKOV3 pada fasa S dan G_2/M. Aktiviti sitotoksik bagi *P. watsonii* yang endemic dan dikutip secara terus dari Taman Negara Endau-Rompin, Johor ke atas sel MCF7, SKOV3, CaSki dan HT29 dikaji buat kali pertama. Kajian menunjukkan bahawa *P. watsonii* mempunyai kesan sitotoksik yang kuat dengan meningkatkan kematian sel secara apoptosis, meningkatkan pengaktifan caspase-3, dan menyebabkan perencatan sel kanser pada fasa berbeza. Dengan itu, *P. watsonii* berpotensi untuk dieksploitasikan selanjutnya dalam pencarian dan pembangunan antikanser farmaseutikal yang baru.

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R.Sujatha
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
µl	microlitre
µm	micrometer
µg/ml	microgram per millilitre
µM	micromolar
ACE	angiotensin-converting enzyme
AFRO	World Health Organization Regional Office for Africa
AIDS	Acquired Immunodeficiency Syndrome
AO	acridine orange
Apaf-1	apoptotic protease activating factor 1
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
β	beta
bp	base pair
CDK	cyclin-dependent kinase
Cl^-	chlorine
cm	centimetre
CO_2	carbon dioxide
CoA	coenzyme A
CPP	cysteine protease
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DED	Death effectors domain
DFF	DNA fragmentation factor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EB	ethidium bromide
ED	effective dose
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISA	enzyme-linked immunosorbent assay
EMRO	European Media Research Organization
EndoG	endonuclease G
ER	endoplasmic reticulum
EtOAc	ethyl acetate
FADD	Fas-associated protein with death domain
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GAE	gallic acid equivalent
h	hour

HEPES	N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonoc
HIV	Human Immunodeficiency Virus
HMW	high molecular weight
HPV	Human Papillomavirus
IC	inhibition concentration
id	internal diameter
K^+	kalium
kbp	kilo-base pair
Kcal	kilocalorie
kg	kilogram
L	litre
LC-MS	liquid chromatography-mass spectrometry
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LMW	low molecular weight
LPO	lipid peroxidation
Me_2CO	acetone
MEM	Minimum Essential Medium Eagle
MeOH	methanol
mg	milligram
mg/ml	milligram per litre
min	minute
ml	mililitre
mm	millimetre
MS-MS	mass spectrometers
MTT	methyl tetrazolium
Na^+	sodium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
ND	not determined
nm	nanometer
NR	Neutral Red
^oC	degree Celsius
OD	optical density
PAP	Papanicolaou
PARP	Poly(ADP-ribose) polymerase
PBS	phosphate buffer saline
PDA	photodiode array
pNa	*p*-nitroanilide
ppm	parts per million
RNA	ribonucleic acid
rpm	rotation per minute
RPMI	Rosewell Park Memorial Institute
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SEARO	South East Asia Region
SI	selectivity index
spp	species
SRB	sulforhodamine B

t	time
TLC	thin layer chromatography
TNF	tumour necrosis factor
TUNEL	triphsphate-biotin nick end labelling assay
UV	ultraviolet
v	velocity
v/v	volume per volume
VLDL	very low-density lipoprotein
w/v	weight per volume
w/w	weight per weight
WHO	World Health Organization
WPRO	World Health Organization Western Pacific Region

LIST OF APPENDICES