ABSTRACT

Swietenia macrophylla King is a large mahogany tree growing in the rainforest of Malaysia. Its seeds have been widely used in traditional medicine to treat various diseases. In this study, anticancer properties of *Swietenia macrophylla* were examined for the first time. Solvent extraction yielded crude ethanolic extract (SMCE) which exhibited prominent bioactivites towards various cancer cell lines namely, MCF-7, KB, HepG2, CasKi and HCT116. Thus, SMCE was fractionated into hexane (SMHF), ethyl acetate (SMEAF) and water fraction (SMWF) for further cytotoxic evaluation. The SMEAF was found to be most potent in inhibiting the growth of human colorectal carcinoma cell line (HCT116) yielding an IC_{50} of 35.35 μg/ml.

SMEAF was found to induce oxidative stress in HCT116 as the treated cells demonstrated increased intracellular ROS level. Inversely, intracellular total glutathione level was lowered. The following experiments suggested apoptosis induction in treated cells, as was reflected by aberrant nuclear changes, sub-G1 cells, disruption of mitochondrial membrane potential, externalization of phosphatidylserine along with activation of the caspase -3/7 and -9. Besides, exposure to SMEAF increased expression level of Bax but not Bcl-2 in HCT116 cells. These results suggested that the treatment with SMEAF may have led the cells to undergo oxidative stress and followed with apoptosis through the intrinsic pathway. Meanwhile, cell cycle analysis in treated cells showed dose-dependent increase in G1 phase population. PCNA Q-PCR analysis confirmed the slowing down of cell replication process upon treatment. The decrease in various cyclin-dependent kinases encoding genes along with the increase in cyclin-dependent kinase inhibitor 1 (p21) and p53 suggested that the arrest of cells might be activated through p53-dependent pathway.

Chemical characterization of fractionated SMEAF by using GC-MS enables identification of the presence of chemical constituents. Compound isolation and structural elucidation of the bioactive SMEAF yielded eight compounds which were mostly limonoids, namely, 3,6-*O,O*-Diacetylswietenolide (1), Swietenine (2), 3-*O*-Tigloylswietenolide (3), Swietenolide (4), Hexadecanoic acid (5), Proceranolide (6), Khayasin T (7) and 6-O-acetylswietenolide (8). Some of the named chemicals (3, 5, 6, 7) were shown to possess cytotoxic effect. Among them, compounds 5, 6, 7 were known apoptotic inducers; the amount of compound 3 was insufficient for in-depth studies. Thus, the focus was shifted to swietenine, a known hypoglycemic inducing agent which shares the same functional groups with compound 3. Chemical modification was performed on swietenine to obtain its derivative that is able to dissolve in aqueous condition for further biological evaluations.

The swietenine derivative, namely swietenine acetate was found to inhibit the growth of HCT116 cell line. This investigation revealed the ability of swietenine acetate to cause apoptosis and arrest of cells. These observed effects were further confirmed using numerous molecular biological techniques. Interestingly, the viable cell population detected in Annexin V/PI did not show further reduction in viability after prolonged exposure to swietenine acetate. This was accompanied by HSP70 protein elevation in swietenine acetate treated cells, which may be responsible for apoptosis impediment in HCT116.

Conclusively, these finding suggested limonoids present in *S. macrophylla* was associated with apoptotic induction in cancer cells. This provides the scientific evidence for the possible use of limonoids derived from *S. macrophylla* for chemoprevention.

ABSTRAK

Swietenia macrophylla King adalah tumbuhan pokok mahogany besar yang boleh didapati dalam hutan Malaysia. Berbijinya digunakan dengan meluas dalam perubatan tradisional. Kajian ini memaparkan kali pertama dalam penyelidikan potensi anti-kanser *Swietenia macrophylla*. Pengesktrakan pelarut menghasilkan ekstrak mentah ethanol (SMCE) yang memberi kesan menonjol terhadap sel-sel kanser seperti berikut, MCF-7, KB, HepG2, CasKi and HCT116. Oleh demikian, SMCE dipecahkan secara berperingkat dan menghasilkan pecahan heksana (SMHF), etil acetat (SMEAF) dan air (SMWF) untuk penilaian kesan sitotoksik yang selanjutnya. Eksperimen menunjukkan pecahan SMEAF sebagai pecahan yang paling kuat dalam menghalang pertumbuhan sel kanser kolorektal (HCT 116) dengan mencatatkan 35.35 μg/ml bagi nilai IC_{50}.

Selain itu, SMEAF menunjukkan keupayaan dalam mendorong pembentukkan tekanan oksidatif dalam HCT116 yang dibuktikan dengan pengesanan dalam peningkatan tahap ROS dan kemerosotan tahap GSH. Ekperimen seterusnya mencadangkan pengaktifan process apoptosis dalam sel yang terdedah kepada SMAF, ini ditunjukkan oleh perubahan nuklear yang tidak normal, sel sub-G1, gangguan potensi membrane mitokondria, pendedahan phosphatidylserine membrane seiring dengan pengaktifan Caspase -3/7 and -9. Selain itu, pendedahan SMEAF meningkatkan tahap Bax dan bukan Bcl-2. Keputusan mencadangkan SMEAF akan menyebabkan sel-sel cancer mengalami tekanan oksidatif diikuti dengan cetusan proses apoptosis melalui laluan intrinsic. Sementara itu, analisis kitaran sel menunjukkan penahanan sel di fasa G1. Analisis PCNA Q-PCR mengesahkan perlahanan replikasi sel akibat pendedahan SMEAF. Tahap kinases dalam kitaran sel juga dirangsangi terutamanya p21dan protein p53 seterusnya mencadangkan penahanan sel melalui laluan p53.

Pencirian kimia pecahan SMEAF dilakukan dengan pengunaan GCMS. Lapan sebatian kebanyakkan adalah limonoids dihasilkan daripada pengasingan SMEAF. Sebatian tersebut termasuk, 3,6-*O,O*-diacetylswietenolide (1), swietenine (2), 3-*O*-tigloylswietenolide (3), swietenolide (4), hexadecanoic acid (5), proceranolide (6), khayasin T (7) and 6-O-acetylswietenolide (8). Beberapa sebatian yang dinamakan seperti 3, 5, 6, 7 menujukkan kesan sitotoksik terhadap sel. Di kalangannya, sebatian 5, 6, 7 adalah pencetus apoptosis yang dikenali; jumlah sebatian 3 terlalu sedikit bagi penyelidikan yang mendalam. Jadi, tumpuan beralih kepada swietenine, salah satu ejen pencetus hipoglisemik yang berkongsi kumpulan berfungsi dengan sebatian 3. Pengusuaian kimia dilakukan terhadap sebatian swietenine untuk mendapatkan derivatifnya yang dapat berlarut sempurna dalam keadaan akueus untuk penilaian biologi yang lebih melanjut.

Derivatif swietenine, swietenine asetat didapati boleh menghalang pertumbuhan sel HCT116. Eksperimen menunjukkan keupayaan swietenine asetat mencetuskan apoptosis dan penahanan kitaran sel disahkan dengan teknik-teknik biologi molekul. Seterusnya, populasi sel yang berdaya yang dikesan dengan Annexin V/PI tidak menunjukkan perubahan yang mendadak dengan pendedahan swietenine asetat yang berlanjutan. Ini membawa kepada penemuan ketingkatan tahap protein HSP70 dalam sel yang terdedah kepada swietenine asetat yang dianggap bertanggungjawab terhadap pencegahan apoptosis dalam sel HCT116.

Kesimpulannya, keputusan yang didapati mencadangkan kehadiran sebatian limonoid dalam *S. macrophylla* dikaitkan dengan pencetusan apoptosis dalam sel-sel kanser. Kajian ini memberi penjelasan saintifik bagi kemungkinan penggunaan sebatian limonoid dari *S. macrophylla* dalam bidang chemopreventive pada masa depan.

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LIST OF SYMBOLS AND ABBREVIATIONS

1D: One Dimensional

13C-NMR: Carbon Nuclear Magnetic Resonance

1H-NMR: Proton Nuclear Magnetic Resonance

3’-OH: 3’ hydroxyl

ACTB: Beta-actin

AIF: Apoptosis inducing factor

Apaf-1: Apoptotic protease-activating factor 1

AR: Analytical reagent

ATCC: American type cell culture

A-T: Adenine- Thymine

ATM: Ataxia telangiectasia mutated

ATP: Adenosine 5’- triphosphate

BAD: Bcl-2- associated death promoter

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2 associated X protein

Bcl-2: B-cell leukemia/ lymphoma 2

Bcl-$_X$L: B-cell lymphoma-extra large

BD: Becton Dickinsion

BH3: Bcl-2 homologoy domain 3

Bid: BH-3 interacting domain death agonist

Bik: Bcl-2 interacting killer

Bim: Bcl-2 interacting mediator of cell death

Bp: Base pair

br: Broad

BRCA: Breast cancer type 1 susceptibility protein

BrdU: Bromodeoxyuridine

Br-dUTP: Bromo-deoxyuridine triphosphate

BSA: Bovine serum albumin

$CaCl_2$: Calcium chloride

CASP8: Caspase 8 encoded gene

Ca Ski: Human cervical carcinoma cell line

Caspase: Cysteine-dependent aspartate-directed protease

CC: Column chromatography

CCNE1: G1/S-specific cyclin E-1

CDC2: Cell division cycle 2

CDC25A: Cell division cycle 25 homolog A

CDK2: Cyclin-dependent kinase 2

CDK4: Cyclin-dependent kinase 4

CDKN1A: Cyclin-dependent kinase inhibitor 1

CDKN2A: Cyclin-dependent kinase inhibitor 2A

CDKs: Cyclin dependant kinases

CFLAR: CASP8 and FADD-like apoptosis regulator

$CHCl_3$: Chloroform

CHEK2: CHK2 checkpoint homolog encoded gene

cm: Centrimeter

CO_2: Carbon dioxide

COSY: Correlation spectroscopy

Ct: Cycle threshold

Cytobuster: Lysis buffer

d: doublet

dATP: Deoxyadenosine 5'- triphosphate

DCFH-DA: 2',7'-dichlorfluorescein-diacetate

DEPT: distortionless enhancement by polarization transfer

DIABLO: Direct inhibitor of apoptosis binding protein with low pi

DMEM: Dulbecco's Modified Eagle's Medium

DMF: Dimethylformamide

DMSO: Dimethyl sulfoxide

DNA: Deoxyribose nuclei acid

DNase: Deoxyribonuclease

dNTP: Deoxyribonucleotide triphosphate

DEPT: Distortionless Enhancement Polarization Transfer

DISC: Death inducing signaling complex

DR: Death receptor

DSB: DNA strand break

DTNB: 5,5-dithio-bis(2-nitrobenzoic acid)

EA: Ethyl acetate faction

E2F1: Transcription factor E2F1

EDTA: Ethylenediaminetetraacetic acid

ERK: Extracellular-regulated kinase

et al: and others

EtOAc: Ethyl acetate

EtOH: Ethanol

F: Fraction

FACS: Fluorescence-activated cell sorting

FADD: Fas-associated death domain

FAS: FAS receptor

Fas L: Fas ligand

FBS: Fetal bovine serum

Fig: Figure

FITC: Fluorescein isothiocyanate

FL-1/ FL-2: Fluorescence channel1/ 2

g: Gram

G1 phase: Gap 1 phase

G2 phase: Gap 2 phase

GADPH: Glyceraldehyde 3-phosphate dehydrogenase

GC-MS: Gas chromatography- mass spectrometry

GR: Glutahione reductase

GSH: Reduced glutathione

GSSG: Oxidized glutathione, glutathione disulfate

GZMA: Granzyme A

h: Hours

HCl: hydrogen chloride

HCT116: Human colorectal carcinoma cell line

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hep G2: Human hepatocellular carcinoma cell line

HMBC: Heteronuclear multiple-bond correlation spectroscopy

Hoechts: Blue fluorescent dye

HPLC: High-performance liquid chromatography

HRMS: High resolution mass spectrometry

HSP: Heat shock protein

HSQC: Heteronuclear single quantum coherence spectroscopy

HTATIP2: Oxidoreductase enzyme

Hz: Hertz

H_2O: Water

IC_{50}: 50% inhibitory concentration

J: Spin-spin coupling constant in Hz

JC-1: 5, 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

KB: Human nasopharyngeal carcinoma cell line

KDa: Kilo dalton

IgG: Immnoglobulin G

LCMS: Liquid chromatography mass spectrometry

m: Multiplet

m: Meter

M phase: Mitotic phase

MCF-7: Human breast carcinoma cell line

MDM2: negative regulator of p53

MeOH: Methanol

mg: milligram

$MgCl_2$: Magnesium chloride

min: minutes

ml: mililitre

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

m/z: Mass to charge ratio

NaCl: Sodium chloride

NADPH: Nicotinamide adenine dinucleotide phosphate

$NaHCO_3$: Sodium bicarbonate

NF-kB: Nuclear fractor- kappa B

NMR: Nuclear Magnetic Resonance

NOESY: Nucler overhauser effect spectroscopy

p21: Cyclin-dependent kinase inhibitor

p53: protein 53 kilodaltons in size

PARP: Poly (ADP-ribose) polymerase

PBGD: Porphobilinogen deaminase

PBS: Phosphate-buffered saline

PCNA: Proliferating cell nuclear antigen

PI: Propidium Iodide

PI3K-Akt: Phosphatidylinositol 3-Kinase/Akt

ppm: parts per million

pRB: Retinoblastoma protein

PS: phosphatidylserine

PTLC: Preparatory Thin Layer Chromatography

q: Quadruplets

Q-PCR: Quantitative polymerase chain reaction

RB1: Retinoblastoma protein

RPMI: Roswell Park Memorial Institute

RNA: Ribonucleic acid

RNase: Ribonuclease

ROS: Reactive oxygen species

Rpm: Rounds per minute

s: single

s: Second

S100A4: Protein S100-A4 containing 2EF-hand calcium-binding motif

S phase: DNA synthesizing phase

SAR: Structure-activity relationship

SDS-PAGE: Sodiun dodecyl sulphate polyacrylamide gel electrophoresis

S.E.: Standard error

SMAC: Second mitochondrial activator of caspase

SMCE: *S. macrophylla* crude ethanolic extract

SMEAF: *S. macrophylla* ethyl acetate fraction

SMHF: *S. macrophylla* hexane fraction

SMWF: *S. macrophylla* water fraction

SSA: 5-sulfosalicyclic acid

t: Triplet

TEMED: Tetramethylethylenediamine

TERT: Telomerase reverse transcriptase

TBE: Tryphan blue exclusion

Tdt: Terminal deoxynucleotidyl transferase enzyme

TLC: Thin Layer Chromatography

TMS: Tetramethylsilane

TNF-α: Tumor necrosis factor receptor

TNFRSF1A: Tumor necrosis factor receptor superfamily ember 1A

TNFRSF10B: Tumor necrosis factor receptor superfamily member 10B

TNFRSF25: Tumor necrosis factor receptor superfamily member 25

TP53: p53 encoded gene

TRAIL: TNF-related apoptosis-inducing ligand

TRIS: tris(hydroxymethyl)aminomethane

Triton X-100: polyethylene glycol tert-octylphenyl ether

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

UV: Ultraviolet

v: Voltage

v/v: Volume to volume ratio

μg: microgram

μl: microliter

μM: micromolar per liter

℃: Degree Celcius

δ: NMR chemical shift in ppm

Δψm: Mitochondrial membrane potential

%: Percent

λ: Wavelenght

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