THE INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING ANTI-TUMOUR AND ANTI-NEUROINFLAMMATION INDUCED BY *Elephantopus scaber* IN *IN VITRO* AND *IN VIVO* MODELS

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FACULTY OF SCIENCE
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Matric No: SHC

Name of Degree: Doctor of Philosophy

Title of Thesis: The investigation of molecular mechanisms underlying anti-tumour and anti-neuroinflammation induced by *Elephantopus scaber* in *in* vitro and *in vivo* models

Field of Study: Biochemistry

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THE INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING ANTI-TUMOUR AND ANTI-NEUROINFLAMMATION INDUCED BY *Elephantopus scaber* IN *IN VITRO* AND *IN VIVO* MODELS

ABSTRACT

Elephantopus scaber L. is a perennial herb which is traditionally used for the treatment of various diseases including hepatitis, cancer, inflammation and eczema. Enormous attention has been drawn among the researchers to ascertain its multifarious pharmacological benefits especially anticancer. Considerable evidence has revealed that *E. scaber* exerts anticancer effect against several cancer cells. In this study, the anti-tumour and anti-neuroinflammatory effects of *E. scaber* leaves and the plausible underlying molecular mechanisms were elucidated using *in vitro* and *in vivo* models. The ethanol extract, hexane, ethyl acetate and aqueous fractions of *E. scaber* exhibited prominent cytotoxicity towards HCT116 and HT-29 colorectal cancer cells. *E. scaber* ethyl acetate fraction (ESEAF) revealed the most potent effect against HCT116 cells (IC_{50}= 1.42 µg/mL). Induction of apoptosis by ESEAF resulted in the anti-proliferative effect as evidenced by the morphological and biochemical characteristics: nuclear shrinkage, chromatin condensation, DNA fragmentation and phosphatidylserine externalization. ESEAF potentiated the ROS generation and p53 activation leading to mitochondrial membrane potential depolarization. This consequently resulted in caspases cascade (caspase-3/7 and -9) and cleavage of poly (ADP-ribose) polymerase eventually apoptosis. Besides, N-acetyl-L-cysteine (NAC) abrogated the production of ROS and reversed the ESEAF-induced apoptosis implying that ESEAF mediated ROS-dependent intrinsic apoptosis in HCT116 cells. Bioassay-guided approach has led to the isolation of deoxyelephantopin (DET) from ESEAF which remarkably inhibited the growth of HCT116 cells. DET was discovered to induce cell cycle arrest at S phase which was modulated by a concerted action of cell cycle related-proteins including p21, p53, CDK2,

CDK4, cyclin D1, A2, E2 and B1. Similar to ESEAF, a ROS inducing agent, DET dysregulated the Bcl-2 family members accompanied by the attenuation of the XIAP and survivin release and the activation of caspase cascades (caspase-8, -10, -9 and -3) implying the involvement of intrinsic and extrinsic pathways. Moreover, mitigation of PI3K/Akt pathway and activation of MAPK pathway were conferred by DET. Furthermore, the event of autophagy occurred upon ESEAF treatment as corroborated by the accumulation of LC3B-II and p62 degradation. In the presence of NAC, HCT116 cells were rescued from apoptosis and autophagy. Importantly, DET prominently suppressed HCT116 tumour growth on the mouse xenograft model which corresponded with the *in vitro* findings. In addition, the anti-neuroinflammatory effect of *E. scaber* leaves were investigated in lipopolysaccharide (LPS)-induced microglia cells (BV-2). In acute toxicity assay, oral administration of 2000mg/kg ESEAF did not result in any mortalities and adverse effects which were substantiated by the normal histopathological evidences. ESEAF also attenuated NF-κB translocation leading to the mitigation of the LPS-induced nitric oxide, inducible nitric oxide synthase, cyclooxygenase-2 and prostaglandin E2 production. The production of pro-inflammatory mediators was suppressed via the inhibition of p38. Upon ESEAF treatment, the formation of ROS in LPS-stimulated BV-2 cells was found to decline following the activation of Nrf2 and HO-1 in which it promotes the scavenging activity of antioxidant enzymes and thus ameliorates neuroinflammation. Conclusively, cumulative findings provided the insight for the potential of *E. scaber* to be developed as nutraceutical for the intervention of cancer and neuroinflammatory disorders.

Keywords: *Elephantopus scaber*, anti-tumour, colorectal carcinoma, anti-neuroinflammation, microglia

KAJIAN MEKANISME MOLEKUL ANTI-TUMOR DAN ANTI-KERADANGAN SARAF DIINDUKSIKAN OLEH *Elephantopus scaber* DALAM MODEL *IN VITRO* DAN *IN VIVO*

ABSTRAK

Elephantopus scaber L. merupakan tumbuhan perennial yang digunakan secara tradisional untuk merawat pelbagai penyakit termasuk hepatitis, kanser, keradangan dan ekzema. Tumbuhan tersebut telah menarik perhatian di kalangan penyelidik untuk memastikan manfaat farmakologi yang berbagai terutamanya antikanser. Banyak bukti telah menunjukkan keberkesanan *E. Scaber* terhadap beberapa sel kanser. Dalam kajian ini, kesan anti-tumour dan anti-keradangan saraf bagi daun *E.scaber* berserta mekanisme molekul yang berkemungkinan telah dijelaskan dalam model *in vitro* dan *in vivo*. Ekstrak etanol, fraksi heksana, etil asetat dan akueus daripada *E. Scaber* mempamerkan sitotoksisiti yang ketara terhadap sel-sel kolon rektum kanser HCT116 dan HT-29. Fraksi etil asetat daripada *E. Scaber* (ESEAF) menunjukkan kesan yang amat poten terhadap sel HCT116 (IC_{50}= 1.42 µg/mL). Induksi apoptosis oleh ESEAF mengakibatkan kesan anti-proliferatif yang dibuktikan melalui ciri-ciri morfologi dan biokimia berikut: pengecutan nuklear, kondensasi kromatin, serpihan DNA dan pendedahan fosfatidilserina. ESEAF mencetuskan penjanaan spesies oksigen reaktif dan pengaktifan p53 membawa kepada penyahkutuban potensi membran mitokondria. Seterusnya ini mengakibatkan pengaktifan kaskad kaspase (kaspase-3/7 dan kaspase-9) dan belahan poli(ADP-ribosa) polimerase (PARP) yang akhirnya mencetuskan apoptosis. Selain daripada itu, N-asetil-L-sisteina (NAC) merencat penjanaan ROS dan membalikkan proses apoptosis yang diinduksikan oleh ESEAF menunjukkan bahawa ESEAF mencetuskan apoptosis intrinsik-bersandarkan ROS dalam sel HCT116. Pendekatan pengasingan berpandukan bioesei telah menghasilkan pengasingan deoxyelephantopin (DET) dari ESEAF yang menghalang pertumbuhan sel HCT1116 dengan amat berkesan. DET didapati

mencetuskan penahanan kitaran sel pada fasa S yang termodulat oleh protein-protein yang berkaitan dangan kitaran sel termasuk p21, p53, CDK2, CDK4, cyclin D1, A2, E2 and B1. Sepertimana ESEAF, suatu ejen pendorong ROS, DET disregulasi ahli keluarga Bcl-2 diiringi oleh penurunan pembebasan XIAP dan survivin serta pengaktifan kaskad kaspase (caspase-8, -10, -9 and -3) mencadangkan penglibatan laluan intrinsik dan extrinsik. Tambahan pula, penyekatan laluan PI3K/Akt dan pengaktifan laluan MAPK telah dirangsangkan oleh ESEAF. Selain itu, autofagi juga berlaku selepas rawatan dengan ESEAF sebagaimana disokong oleh pengumpulan LC3B-II dan degradasi p62. Dengan kewujudan NAC, sel HCT116 telah diselamatkan daripada apoptosis dan autofagi. Yang pentingnya, DET menyekat pertumbuhan tumor HCT116 pada model tikus xenograft yang selari dengan penemuan *in vitro*. Di samping itu, kesan anti-keradangan saraf oleh daun *E. scaber* telah dikaji pada sel mikroglia (BV-2) yang diinduksikan oleh liposakarida. Dalam esei ketoksikan akut, pengambilan 2000g/kg ESEAF secara oral tidak membawa sebarang kematian dan kesan buruk yang dibuktikan oleh histopatologi yang normal. ESEAF juga mengurangkan translokasi NF-κB yang membawa kepada penyekatan keatas penghasilan nitrik oksida, nitrik oksida sintase teraruhkan, siklooksigenase-2 (COX-2) dan prostaglandin E_2 yang diinduksi oleh LPS. Penghasilan pengantara pro-keradangan dihalang melalui perencatan p38. Apabila dirawat dengan ESEAF, pembentukan ROS dalam sel BV-2 yang dirangsangkan oleh LPS didapati menurun berikutan pengaktifan Nrf2 dan HO-1 yang mendorong aktiviti memerangkap oleh enzim antioksidan, dengan itu keradangan saraf dikurangkan. Kesimpulannya, hasil kajian yang kumulatif ini telah memberi pengetahuan mengenai potensi *E. scaber* sebagai nutraseutikal khasnya bagi rawatan kanser dan penyakit keradangan saraf.

Kata kunci: *Elephantopus scaber*, anti-tumor, kolon rectum kanser, anti-keradangan saraf, mikroglia

ACKNOWLEDGEMENTS

This thesis represents not only my work at the keyboard, it is a milestone and breakthrough in the fields of molecular oncology and biochemistry. To have succeeded this study created an unforgettable life experience for me and it would not have been possible to do without the support and guidance that I received from this remarkable individuals who I wish to acknowledge. First and foremost I would like to express my enormous gratitude to my supervisor Professor Dr. Habsah Abdul Kadir who shapes who I am. It is truly my proud privilege to work under her, as her unwavering enthusiasm she has for her research was always contagious and motivational for me throughout my study. Without her insightful comments and immeasureble guidance, this PhD would not have been achievable. My sincere gratitude also devoted to the support I received from the collaborative work I undertook at the Department of Chemistry, Faculty of Science and Department of Pharmacology, Faculty of Medicine, especially Professor Dr Khalijah Awang, Dr Chan Gomathi, Associate Professor Dr. Wong Pooi Fong and Tong Kind Leng for their assistance and guidance in my bioactive compound isolation and *in vivo* anti-tumour study. Besides, I would also like to express my sincere thanks to Professor Dr. Noor Hasima Nagoor Pitchai and Phuah Neoh Hun for their generosity and assistance in providing the indispensable laboratory facilities to complete my study. My appreciation also extends to Dr Goh Bey Hing, Shathiswaran N Andy, Ahmand Fdhlurrahman Ahmad Hidayat and Wong Kar Suen for the valuable assistance. I would like to acknowledge University of Malaya for awarded Bright spark Scholarship and University Malaya Postgraduate Research Fund (PG053-2015A) to make my PhD possible. Above ground, I'm indebted to my lovely parents, Mr Chan Yeow Moon, Mrs Chan Huang Siew Cho and my spouse, Mr Lee Woon Chung for their unconditional loves and unfailing motivation throughout the journey.

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LIST OF SYMBOLS & ABBREVIATIONS

α	:	Alpha
β	:	Beta
°C	:	Degree celcius
C	:	Carbon
δ	:	Delta
γ	:	Gamma
g	:	Gram
h	:	Hour
H	:	Hydrogen
kDa	:	Kilodalton
kg	:	kilogram
mg	:	Milligram
min	:	Minute
mL	:	Millilitre
mM	:	Millimolar
µM	:	Micromolar
µg	:	Microgram
µL	:	Microlitre
$\Delta\psi m$	:	Mitochondrial membrane potential
Na_2CO_3	:	Sodium bicarbonate
S.E.	:	Standard error
Aβ	:	β-amyloid
AD	:	Alzheimer's disease
AIF	:	apoptosis-inducing factor

ALP	:	alkaline phosphatase
ALT	:	alanine aminotransferase
AO	:	acridine orange
Apaf-1	:	apoptotic protease-activating factor 1
ARE	:	antioxidant response element
AST	:	aspartate aminotrasferase
Atg	:	autophagy
AVO	:	acidic vesicular organelle
Bax	:	Bcl-2-associated X protein
BBB	:	Blood Brain Barrier
Bcl-2	:	B cell lymphoma 2
Bcl-xL	:	B cell lymphoma-extra large
CAEE	:	ethyl 3,4-dihydroxycinnamate
CAT	:	catalase
CDK	:	cyclin-dependent kinases
CDKI	:	CDK inhibitors
CNS	:	central nervous system
CO	:	carbon monoxide
COX-2	:	cyclooxygenase-2
CRC	:	Colorectal cancer
CRE	:	creatinine
DCFH-DA	:	2’7’-dichlorofluorescein diacetate
DR4	:	Death receptor 4
DR5	:	Death receptor 5
DET	:	deoxyelephantopin
Diablo	:	direct inhibitor of apoptosis protein (IAP)-binding protein with low PI

DISC	:	death-inducing signalling complex
DMEM	:	Dulbecco’s Modified Eagle’s Medium
DMSO	:	Dimethyl sulphoxide
ECL	:	enhanced chemiluminescence
EMEM	:	Eagle's Minimum Essential Medium
eNOS	:	endothelial nitric oxide synthase
ERK	:	extracellular signal-regulated protein kinases
ESAF	:	*E. scaber* leaves' aqueous fraction
ESEAF	:	*E. scaber* leaves' ethyl acetate fraction
ESEE	:	*E. scaber* leaves' ethanol extract
ESHF	:	*E. scaber* leaves' hexane fraction
FADD	:	Fas associated death domain
FasL	:	Fas ligand
FBS	:	Fetal bovine serum
FU	:	fluorouracil
HO-1	:	heme oxygenase-1
H_2O_2	:	hydrogen peroxide
GC-MS	:	Gas chromatography-mass spectrum
GPx	:	Glutathione peroxidase
G6PD	:	glucose-6-phosphate dehydrogenase
GSSG	:	glutathione disulphide
HEPES	:	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IAPs	:	inhibitor apoptosis proteins
IC_{50}	:	Inhibitory concentration at 50%
ICAD	:	including inhibitor of caspase activated DNAse
IGFBP	:	insulin-like growth factor binding proteins

IGF2R	:	insulin-like growth factor 2 receptor
IKK	:	IκB kinase
IL	:	interleukin
IL-1β	:	interleukin-1β
IL-6	:	interleukin-6
i.p.		intraperitoneal
iNOS	:	inducible nitric oxide synthase
iso-DET	:	isodeoxyelephantopin
JNK	:	stress-activated c-Jun NH2-terminal kinase
Keap1	:	Kelch-like ECH-associated protein-1
LC3	:	Microtubule-associated protein 1 light chain 3
LPS	;	lipopolysaccharide
LV	:	leucovorin
MAPK	:	mitogen-activated protein kinase
MOMP	:	mitochondrial outer membrane permeabilisation
mTOR	:	mammalian target of rapamycin
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	:	N-acetylcysteine
NADPH	:	nicotinamide adenine dinucleotide phosphate
Nrf2	:	nuclear factor erythroid 2-related factor 2
nNOS	:	neuronal nitric oxide synthase
NO	:	nitric oxide
NF-κB	:	Nuclear factor k-light-chain-enhancer of activated B cells
PARP	:	poly(ADP-ribosyl) polymerase
PBS	:	Phosphate buffer

PCNA	:	proliferating cell nuclear antigen
PDK1	:	phosphoinositide-dependent kinase 1
PGE_2	:	prostaglandin E_2
PI	:	propidium iodide
PI3K	:	Phosphoinositide-3-kinase
PI3P	:	phosphatidylinositol 3,4,5-triphosphate
PTEN		Phosphatase and TENsin homolog deleted on chromosome 10
RIPA	:	radioimmunoprecipitation assay
RNS	:	reactive nitrogen species
ROS	:	reactive oxygen species
RPMI 1640	:	Roswell Park Memorial Institute 1640
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	:	superoxide dismutase
STAT	:	signalling transducer and activator of transcription 3
TBHP	:	tert-butyl hydroperoxide
TBIL	:	total bilirubin levels
TEM	:	transmission electron microscopy
TEMED	:	Tetramethylethylenediamine
TGF-βRII	:	transforming growth factor β receptor II
TNF	:	tumour necrosis factor
TNF-α	:	tumour necrosis factor-α
TNFR1	:	Tumour necrosis factor receptor 1
TNFR2	:	Tumour necrosis factor receptor 2
TRAIL-R1	:	Tumour necrosis factor related apoptosis inducing ligand receptor 1
TRAIL-R2	:	Tumour necrosis factor related apoptosis inducing ligand receptor 2

TUNEL	:	Terminal Deoxynucleotidyl Transferase UTP Nick End Labeling
ULK-mAtg13-FIP200	:	UNC-51-like kinase complex
XIAP	:	X-linked inhibitor of apoptosis

CHAPTER 1: INTRODUCTION

Colorectal cancer is the third most diagnosed malignancy in the world. In spite of the latest advances in the cancer treatment approach, the malignancy remains threatening to humans pertaining to the ineffectiveness of the conventional approaches such as chemotherapy, radiotherapy and surgery (Torre *et al.*, 2015). Therefore in order to overcome these circumstances, discovery of new approaches based on natural products is of great interest and need among the researchers.

A diverse of cell death modalities including apoptosis, necrosis and autophagy which are promising anticancer strategies for the development of therapeutic drugs. Apoptosis machineries which are intrinsic and extrinsic pathways can be governed by a myriad of apoptotic proteins including Bcl-2 family proteins, caspases and p53 (Olsson & Zhivotovsky, 2011). Extrinsic pathway is triggered by the oligomerization between ligands and death receptors whereas intrinsic pathway is mediated via caspase cascades upon activation of death receptor or a plethora of stimuli (Baig *et al.*, 2016; Li, 2013; Pradelli *et al.*, 2010). Activation of intrinsic pathway is triggered by a set of apoptotic substrates including cytochrome c, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein-binding protein with low PI (Smac/DIABLO), endonuclease G, apoptosis-inducing factor and Omi/HtrA2. This is then followed by the release of the inhibitor apoptosis proteins (IAPs) and the activation of caspase cascade further unleashed apoptotic substrate cleaved PARP ultimately led to apoptosis (Li, 2013).

Autophagy takes part in the regulation of homeostasis which is described as a highly conserved cellular degradation process and characterized by the presence of autophagosomes (Fulda & Kogel, 2015). Excessive amounts of ROS can trigger several signalling transductions such as apoptosis, MAPK pathway and autophagy (Ouyang *et*

al., 2012). ROS can induce the activation of MAPK signalling transduction via ERK, JNK and p38 MAPK which leads to apoptosis (Wada & Penninger, 2004). However, dysregulation of mTOR, Akt and NF-κB signalling promotes cancer progression and drug resistance in colorectal cancer. Therefore, the inhibition of multiple survival pathways such as PI3K/Akt/mTOR and NF-κB can be useful in inhibiting tumour growth as well as in restoring the sensitivity of tumour cells to chemotherapeutic drugs (Gupta *et al.*, 2003; Khan *et al.*, 2013; Papadatos *et al.*, 2015).

Neurodegenerative diseases such as Alzheimer's disease, multiple sclerosis and Parkinson's disease that are major health problems are becoming worldwide concern. Neurodegenerative disorders are caused by progressive loss of neuronal cells which are conceivably provoked by the oxidative stress as well as inflammatory response. Enormous body of evidence has reported that neuroinflammation is associated with the neurodegenerative pathogenesis which is orchestrated by microglia (Chen, 2016). Activation of microglia induces neurotoxic effects by causing excessive secretion of the pro-inflammatory mediators including prostaglandin E_2 (PGE_2), cytokines, nitric oxide (NO) and reactive oxygen species (ROS) which ultimately leads to inflammatory response (Amor *et al.*, 2010; Fischer & Maier, 2015; Liu & Hong, 2003). These inflammatory responses are triggered by the activation of p38 MAPK pathway. Besides, the activation of p38 pathway further induces the translocation of NF-κB resulting in production of pro-inflammatory mediators. Hence, NF-κB and p38 are regarded as promising targets for the treatment of neurodegenerative diseases (Munoz & Ammit, 2010; Shih *et al.*, 2015). In addition, oxidative stress is believed to be involved in the progression of neuroinflammation. Thus, antioxidants enzymes including superoxide dismutase (SOD) and catalase as well as antioxidant defence mechanisms such as Nrf2/HO-1 signalling pathway play key roles in targeting the oxidative stress and inflammation to reverse the neuronal death as well to ameliorate the progression of

neurodegenerative diseases (Hsieh & Yang, 2013; Innamorato *et al.*, 2009; Nguyen *et al.*, 2009)

Elephantopus scaber L. commonly known as elephant's foot or "tutup bumi" is widely distributed in various countries in Asia including Malaysia, Sri Lanka, Vietnam, Hong Kong, Taiwan, Philipines, Japan, Thailand, Indonesia, India, Myanmar as well as China, Europe, Africa and America (Ho *et al.*, 2009). *E. scaber* is traditionally used as folk remedies to treat arthritis, hepatoprotective, hepatitis, diarrhoea, cancer, leukaemia, diabetes, fever, insomnia and eczema (Achuta *et al.*, 2010; Hammer & Johns, 1993; Rasoanaivo *et al.*, 1992). It is reported that *E. scaber* has abundant of sesquiterpene lactones including deoxyelephantopin (DET) and isodeoxyelephantopin (iso-DET). Cumulating scientific reports have shown that deoxyelephantopin, one of the major components from *E. scaber* possesses anti-cancer effect on breast, nasopharyngeal, lung and prostate cancer (Gurib *et al.*, 1993; Ho *et al.*, 2009; Lin *et al.*, 1995; Su *et al.*, 2009). Additionally, isodeoxyelephantopin was demonstrated to elicit apoptosis in chronic myeloid leukaemia, breast and lung carcinoma (Ichikawa *et al.*, 2006; Kabeer *et al.*, 2014).

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Aberrant development occurring in cancer is attributed to the successive genetic alterations which progressively evolve the cells from normalcy to malignancy states, often referred as a multistep carcinogenesis. Growing study reported that a skewed ratio in cell survival and cell death unambiguously favours carcinogenesis (Labi & Erlacher, 2015). Thus, carcinogenesis is initiated and continuously progressed via the acquisition of several capabilities including evasion of apoptosis, boundless replication, insensitivity to anti-growth signals, sustained growth signals, persistent angiogenesis, tissue invasion and metastasis, restructuring of energy metabolism and dodging immune destruction. A string of these alterations may lead to the development of hyperplasia, adenoma, carcinoma and metastases (Hanahan & Weinberg, 2000, 2011; Labi & Erlacher, 2015).

2.1.1 Colorectal cancer

Colorectal cancer (CRC), a multifaceted disease is ranked as the third most prevalent malignancy and one of the leading cause of lethality in the world, after prostate/breast cancer and lung (Society, 2017). Despite significant understanding of the colon tumourigenesis, CRC still remains a major concern as displayed by the high mortality and morbidity worldwide (Siegel *et al.*, 2016).

CRC transforms from normal colonic epithelium to adenoma, carcinoma, eventually metastasis and invasion. The pathogenesis of CRC is resulted from the aberrant accumulation of genetic and epigenetic instability which is driven by three distinct pathways: the chromosomal instability pathway, the microsatellite instability pathway and CpG island methylator phenotype. Most CRC primarily executes chromosomal instability pathway which is a classical process characterized by accumulation of

chromosomal abnormalities leading to loss of heterozygosity generating gene deletions, duplications and chromosomal rearrangements. In contrast, microsatellite instability pathway is caused by the deficiency of DNA mismatch repair which is essential in the release of proteins that are able to direct repair the mismatched nucleotides at microsatellite sequences. For microsatellite instability, the frameshift mutation including insertion and deletion mutations are often detected in the repetitive DNA sequences (Jass, 2007). Various genes have been implicated in colon cancer pathogenesis including Bcl-2-associated X protein (Bax), transforming growth factor β receptor II (TGF-βRII), β-catenin and insulin-like growth factor 2 receptor (IGF2R) (Markowitz *et al.*, 1995; Souza *et al.*, 1996; Yamagishi *et al.*, 2016). In addition, CpG island methylator phenotype is described by the emergence of aberrant methylated CpG dinucleotides. The promoter CpG island hypermethylation leads to transcriptional silencing in turn inhibits the tumour suppressors and DNA repair genes, which is an epigenetic alteration that affects the pathogenesis of CRC (Lee *et al.*, 2008). These pathways contribute to the adenoma-carcinoma sequence which transforms normal colonic mucosa into CRC via accumulation of dysfunction tumour suppressor genes and oncogenes (Fearon & Vogelstein, 1990; Yamagishi *et al.*, 2016). Initially, the normal colonic mucosa transforms into polyps via APC mutation followed by the activation of KRAS mutations along with defects in DNA mismatch repair which leads to the formation of adenoma (Yamagishi *et al.*, 2016). Mismatch repair deficiency occurs via downregulation of MLH1 by promoter methylation (Cunningham *et al.*, 1998; Geiersbach & Samowitz, 2011; Imai & Yamamoto, 2008). Subsequently, the accumulation of gene mutations which encompassed Sma- and Mad-related protein 4 (SMAD4), phosphatidylinositol-4,5-bisphosphate 3-kinase, TGFBR2, IGF2R, PTEN, p53 and Bax, are required in the progression of adenoma to carcinoma (Figure 2.1) (Fearon & Vogelstein, 1990; Pereira *et al.*, 2015; White, 1998; Yamagishi *et al.*, 2016).

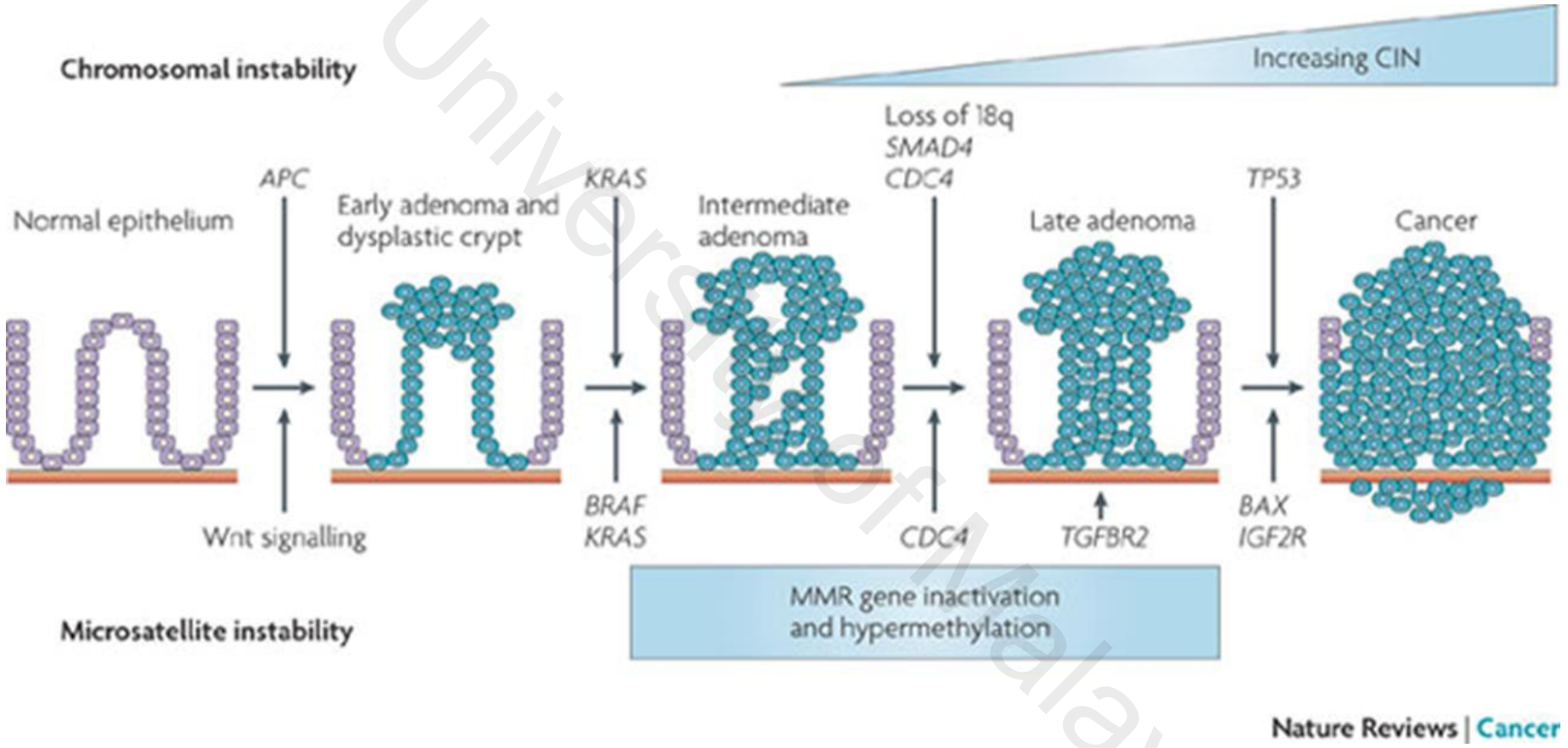

Figure 2.1: Molecular, genetic and epigenetic alterations which involve in the tumourigenesis of colon. Adapted from Walther *et al.* (2009).

2.1.2 Treatment modes for colorectal cancer

In the past two decades, a myriad of cancer therapy strategies are available and tailored for the management of cancer encompassing chemotherapy, surgery, immunotherapy, targeted therapy and radiation therapy. Single or combination treatment modalities will be selected according to the types and stages of colorectal cancer (Mishra *et al.*, 2013). To date, surgical resection and chemotherapy remain mandatory in fighting cancer especially in patients with advanced stage (Lawes & Boulos, 2002; Netter *et al.*, 2016).

In colorectal cancer, the primary treatment modality is resection surgery followed by adjuvant chemotherapy (Society, 2017). The patients with stage I or II colorectal cancer usually undergo surgery which is colectomy. However, surgical resection, a sole treatment modality is often associated with poor survival due to the recurrence or incomplete removal of cancerous cells. Hence, patients who underwent the surgery or at stage III will receive neoadjuvant chemotherapy which often brings deleterious side effects and develops drug resistance in cancer patients. For stage IV patients, chemotherapy is the main treatment either alone or in combination with radiation therapy, before or after surgery to lower the risk of recurrence (Miller *et al.*, 2016). The common chemotherapeutic drugs which have been utilized are fluorouracil (FU), irinotecan, leucovorin (LV) and cetuximab. To increase the efficacy, multi-agent chemotherapy such as irinotecan or oxiplatin with FU/LV is applied to increase the survival rate (Saltz *et al.*, 2000). However, combination therapy other than increasing the efficacy, simultaneously increases the toxicity (Zuckerman & Clark, 2008).

In spite of enormous improvement in chemotherapy modality, treatment results are rather disappointing evidenced by the overall survival rate and mortality of patients. The paradigm for chemotherapy modality has progressed from non-specific cytotoxic agents to selective mechanism based therapeutics. However, these therapeutic drugs which

emerge as a backbone of current treatment are constrained by several factors such as narrow therapeutic index, remarkable toxicities and often along with drug resistance. Therefore, recent advances of the treatment modality is combination treatment such as immunotherapy and targeted therapy together with the conventional methods especially for the advanced stage colorectal cancer patients. (Vanneman & Dranoff, 2012). Nevertheless, there is considerable commercial and scientific interest continuously emerging to discover safer and effective natural-derived compounds for the treatment of cancers. Food and Drug Administration has approved growing numbers of plant-derived anticancer agents which have been extensively proven to mediate diverse cell death machineries and emerged as clinical interventions owing to their efficacy, safety profile and absence of drug resistance. Thus, plants remain as the best source for the discovery and development of natural-derived drugs to combat various dreaded diseases including cancer (Cragg & Newman, 2005, 2013; Unnati, 2013).

2.1.3 Cell death modalities

Cell death contributes in orchestrating the events of tissue homeostasis, embryonic development, infection resistance, immune system and tumour inhibition. Equilibrium between cell survival and cell death is of utmost importance for the aforementioned events. Perturbation of the balance between cell death and survival lead to progression of diverse pathological disease including neurodegenerative diseases, cancer, embryogenesis, autoimmune system and others (Broker *et al.*, 2005). Distinct forms of cell death modalities have been described comprising of apoptosis, necrosis, autophagy, necroptosis and other cellular demise (Kroemer *et al.*, 2009). Programmed cell death mainly refers to apoptosis, autophagy and necrosis is proposed to be the machineries to determine the fate of malignant neoplasm cells (Broker *et al.*, 2005). It has been

increasingly noted that these cell death mechanisms aid in the improvement and development of safer yet effective chemotherapeutic agents for cancer treatment.

2.1.4 Apoptosis

Apoptosis, also known as type I programmed cell death, is an orchestrated machinery that occurs to remove unhealthy or aged cells from the body in order to maintain the development. However, malignant derivatives often dampen the apoptotic response as the malfunction of cell death regulatory circuits prevents effective removal by the intracellular anti-cancer mechanism or the available anti-cancer therapies (Lowe & Lin, 2000).

Dysfunction of apoptosis steers the shift towards tumour formation consequently develops drug resistance. Thus, discovering natural derived-compounds which can effectively induce apoptosis in cancer is regarded as potential chemotherapeutic candidate. Apoptosis can be generally characterized by morphological hallmarks. The apoptotic cells first manifest by the rounding-up of the cell, chromatin condensation and consequently result in reduction of cellular volume (pyknosis). Next, membrane blebbing occurs followed by nuclear fragmentation (karyorrhexis). The contents of cell forms apoptotic bodies which subsequently undergoes engulfment by resident phagocytes (Galluzzi *et al.*, 2007). Furthermore, several biochemical features particularly phosphatidylserine externalization, attenuation of mitochondrial membrane potential and caspase activation can be detected in apoptotic cells.

2.1.4.1 Mechanisms of apoptosis

Apoptosis is a cell demise machineries to eliminate unnecessary and unhealthy cells. It is hampered in most of the common cancers and hence resulting in apoptosis resistance where cancer cells able to reap the advantages for uncontrollable growth and survival.

Apoptosis resistance is mainly attributed to the mutations of several pro-apoptotic proteins such as p53 (Muller & Vousden, 2013) or death receptors (Lee *et al.*, 2001). In other words, by targeting apoptosis resistance could effectively eliminate the cancer cells. Therefore, apoptotic machineries has been well established as one of the approaches for chemotherapy in targeting aberrant proliferation of cancer (Wong, 2011). Apoptosis machinery is tightly regulated by two major pathways which are extrinsic pathway and intrinsic pathway which involving death receptors and mitochondria, respectively. Extensive studies have widely documented the interrelation of intrinsic and extrinsic pathway in mediating apoptotic cell death (Adams, 2003; Fulda, 2015).

2.1.4.2 Extrinsic pathway

Activation of extrinsic pathway requires the ligation of death ligands and death receptors to trigger apoptosis (Adams, 2003; Fulda, 2015). Several cell surface death receptors have been discovered namely tumour necrosis factor related apoptosis inducing ligand receptor 1 (TRAIL-R1), also referred as DR4, TRAIL-2 (also referred as DR5), DR3 (Apo-3), DR6, Fas (CD95) or tumour necrosis factor receptors (TNFRs). Each of the DRs can be activated by the specific ligands upon oligomerization which comprised of Fas ligand (FasL), tumour necrosis factor (TNF-α) as well as TRAIL (Ashkenazi, 2008a). Oligomerization of DRs and ligands complexes with cytoplasmic adapter protein Fas associated death domain (FADD). Consequently, FADD further recruits initiator caspases, procaspase-8 and -10 to form death-inducing signalling complex (DISC). These recruitment further execute a series of proteolytic event via two different ways: directly activates effector caspase-3/-7 or indirectly propagates death signal via activation of mitochondrial-mediated apoptosis/intrinsic pathway by provoking truncated Bid to translocate to mitochondrial membranes and consequently initiates mitochondrial outer membrane permeabilization (Figure 2.2) (Bai & Wang, 2014). This process can be

blocked by c-FLIP (FLICE-inhibitory proteins) which exerts anti-apoptotic effect via inactivating clustering of procaspase-8 to the DISC (Krueger *et al.*, 2001).

2.1.4.3 Intrinsic pathway

Intrinsic pathway or mitochondrial-mediated apoptosis which involves mitochondria is initiated by numerous stimuli including DNA damage, oxidative stress, growth factor or chemotherapeutic drugs (Labi & Erlacher, 2015). The intrinsic pathway is predominantly governed by B cell lymphoma 2 (Bcl-2) family proteins. Regardless of various stimuli induce apoptosis via diverse of signalling transductions, these signals eventually congregate on mitochondria leading to the permeabilisation of mitochondrial outer membrane (MOMP). MOMP is tightly orchestrated by Bcl-2 family proteins which serve as a rheostat in regulating mitochondrial-mediated apoptotic pathway (Volkmann *et al.*, 2014). Cellular stress induces activation of p53 tumour suppressor protein which executes intrinsic pathway by promoting p53 upregulated modulator of apoptosis and NOXA. These in turn result in neutralization of anti-apoptotic Bcl-2 proteins including Bcl-2 and Bcl-extra large (Bcl-xL) which dissociate from pro-apoptotic Bcl-2 proteins including Bax and Bak to initiate the apoptotic signal. Heterodimerisation of Bax and Bak promotes the pore formation leading to MOMP which unleashes pro-apoptotic substrates including cytochrome c and Smac/DIABLO into cytosol (Ashkenazi, 2008b; Fox & MacFarlane, 2016). The release of cytochrome c results in apoptosome formation consists of adaptor protein apoptotic protease-activating factor 1 (Apaf-1) and caspase-9 facilitating the downstream caspase cascades activation (caspase-3/-7). MOMP instigates progressive dysfunction of mitochondria and ultimately lead to cell death such as apoptosis (Suzuki *et al.*, 2000; Volkmann *et al.*, 2014). In contrast, the release of SMAC amplifies apoptosis signal by interrupting the regulation of inhibitor of apoptosis proteins (IAPs). SMAC neutralizes IAP proteins such as X-linked inhibitor of apoptosis (XIAP)

by releasing brake on caspase-9 and -3 in order to promote caspase cascades activation (Fulda & Vucic, 2012). Other than apoptotic stimuli, p53 could directly activate Bax or release the grip of antiapoptotic Bcl-2 family members such as Bcl-xL or Bcl-2 away from Bax. In contrast, anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, function as a prominent role in preventing the induction of apoptosis. Bcl-2 or Bcl-xL binds to Bax and Bak leading to the formation of heterodimers, thereby inhibiting MOMP and conserving mitochondrial integrity (Borner, 2003). It is extensively documented that aberrant regulation of pro- and anti-apoptotic Bcl-2 family members has been found closely associated with tumourigenesis (Strasser *et al.*, 1997). Defects in pro-apoptotic Bcl-2 family members such as Bax and Bak which function as tumour suppressor commonly found in various human malignancy (Yip & Reed, 2008). On the contrary, the anti-apoptotic Bcl-2 family members have demonstrated to cause transformation of oncogenic cells from normal cells thereby enhancing the survival of the cancerous cells. However, overexpression of anti-apoptotic Bcl-2 family in concerted with the mutations as such deletion of p53 promotes cancer cell proliferation (Hanahan & Weinberg, 2000; Yip & Reed, 2008). Therefore, modulating the anti- and pro-apoptotic Bcl-2 proteins which confer apoptosis can be exploited as one of the potential strategies to target tumourigenesis

Both the extrinsic and intrinsic pathways induce executioner caspase-3 activation and carry out the apoptotic execution via cleavage of cellular substrates including inhibitor of caspase activated DNAse, actin, poly ADP ribose polymerase (PARP), cytokeratins, fodrin and others. These ultimately result in apoptotic cell death with the observations of biochemical and morphological alterations comprising of DNA fragmentation, cellular shrinkage and phosphatidylserine externalization (Figure 2.3) (Slee *et al.*, 2001).

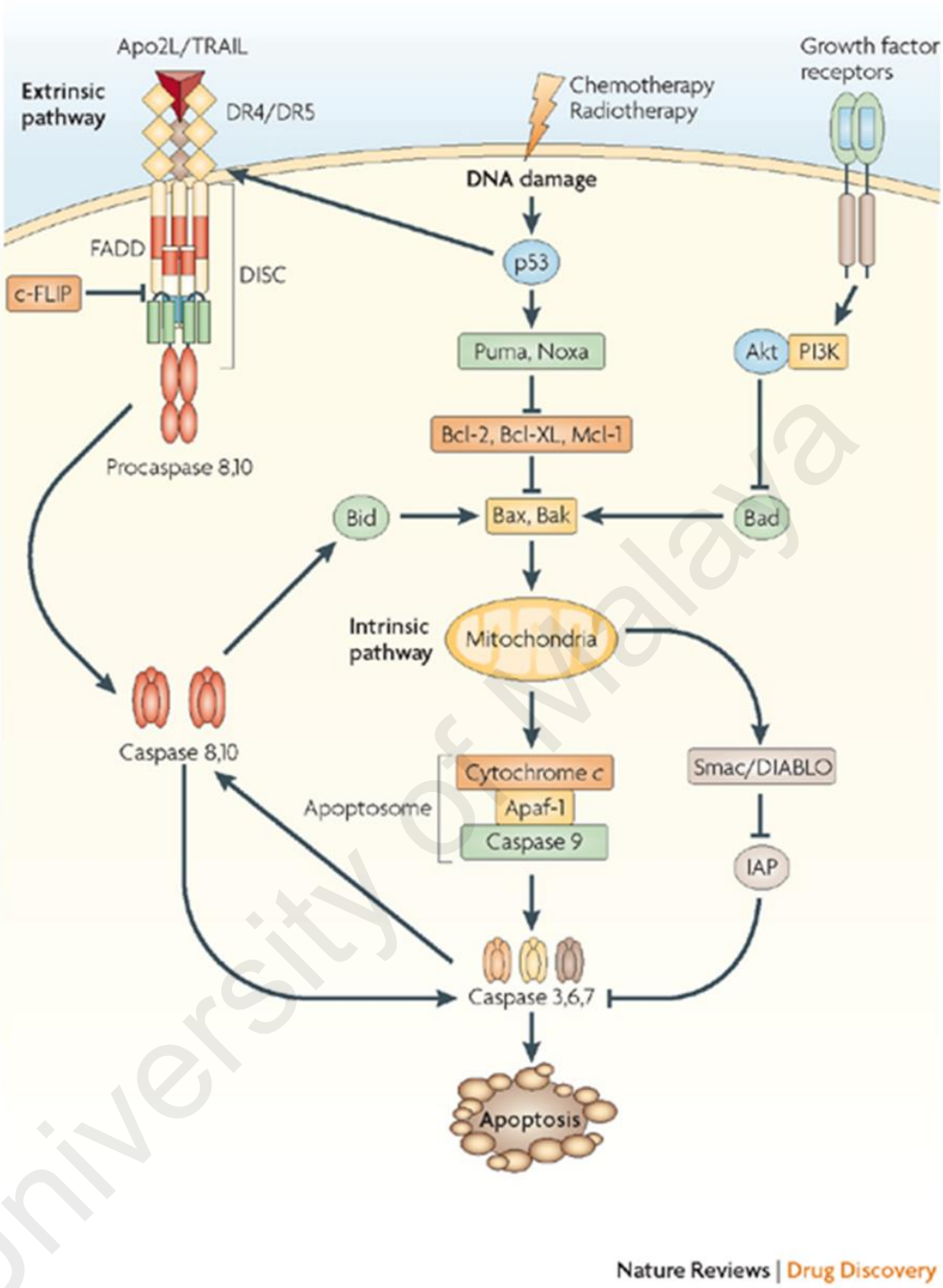

Figure 2.2: Extrinsic and intrinsic apoptotic pathways. Adapted from Ashkenazi (2008a).

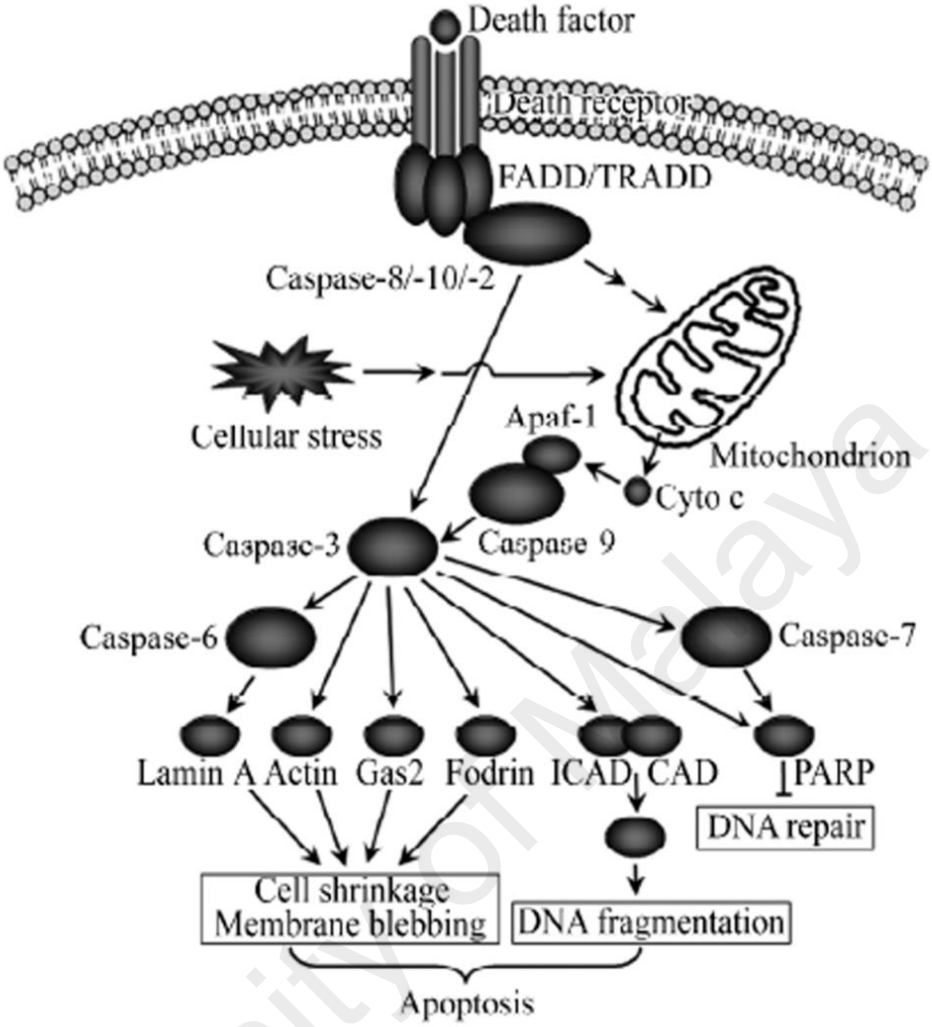

Figure 2.3: The release of downstream apoptotic substrates upon activation of caspase-dependent apoptosis. Adapted from Fan *et al.* (2005).

2.1.5 Necrosis

Apart from apoptosis, another alternative cell death mechanism is necrosis. Apoptosis can be distinguished from necrosis by a few factors such as the degree of ATP depletion and the caspases availability. Necrosis is regarded as an uncontrollable and energy-independent process which influence large fields of cells whereas apoptosis is a controllable and energy-dependent process yet affecting only clusters of cells. (Chaabane *et al.*, 2013).

Morphologically, necrosis is manifested by the features of gain in cell volume (oncosis), cell swelling, disruption of plasma membrane and dismantling swollen organelles including condensed or rupture mitochondria. In contrast, apoptosis is defined by the characteristics including pyknosis, karyorhexis, rounding bodies and formation of apoptotic bodies. Necrosis is regarded as harmful event as it often is related to the pathological cell loss (Galluzzi *et al.*, 2007). In addition, upon the leaking of the cytosolic constituents into the intracellular space via the rupture plasma membrane integrity, inflammatory response will be initiated (Colton & Wilcock, 2010). During apoptosis, the cellular constituents are not released into intracellular space and instead engulfed by phagocytes without the trigger of inflammatory response (Block *et al.*, 2007; Chaabane *et al.*, 2013).

2.1.6 Autophagy

Autophagy is a highly conserved self-cannibalization mechanism which involves engulfment of cellular components consisting of unwanted, impaired and dysfunctional organelles and misfolded proteins via lysosomal degradation pathway (Levine & Kroemer, 2008). It contributes to various pathophysiological processes such as development, cell survival and death, aging, innate and adaptive immunity (Mehrpour *et*

al., 2010). Considerable evidence has reported that autophagy acts as double edge sword which possesses cytoprotective and cytotoxic functions in diseases including cardiovascular diseases, neurodegenerative diseases, diabetes and particularly in cancer (Fulda & Kogel, 2015; Meijer & Codogno, 2009; White & DiPaola, 2009).

Autophagy can be detected by the morphological alterations such as massive vacuolization of the cytoplasm which is also known as double membrane autophagosomes. A plethora of stimuli such as stress, aggregation of protein, chemotherapeutic drugs and organelle dysfunction can trigger the onset of autophagy in which many targeting via the central autophagy regulator mTOR (Fulda & Kogel, 2015). Interactions between autophagosomes and lysosomes involving a concerted action of prominent proteins including autophagy-related proteins (ATG), p62, Beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3) in response to oxidative stress stimuli is manifested as autophagy.

Generally, it can be categorized into two major pathways: canonical pathway which is dependent on Beclin-1 and non-canonical pathway which is independent of Beclin-1. Autophagy, is a multi-step process which can be regulated by various pathways including adenosine monophosphate kinase (AMPK), MAPK, mTOR, PI3Ks and PKC (Hasima & Ozpolat, 2014). mTOR is a negative regulator of autophagy where suppression of mTOR activation results in autophagy. Mechanistically, UNC-51-like kinase complex (ULK-mAtg13-FIP200) is required for the onset of autophagy via activation of AMPK and/or inhibition of mTORC1 as shown in Figure 2.4 (Jung *et al.*, 2009; Kim *et al.*, 2011). Next, Beclin-1 activates Vps34 and Vps15 to trigger the formation of PtdIns3K complex which is required for the nucleation of an isolation membrane, phagophore. Vps34 further induces generation of phosphatidylinositol 3,4,5-triphosphate (PI3P) consequently PI3P recruits proteins that is mandatory for the event of elongation. Elongation and maturation

of autophagosomes is activated via two ubiquitin-like conjugation systems which involving a series of ATG proteins: (1) ATG5, ATG12 and ATG16, and (2) ATG7 and ATG3 (Fulda & Kogel, 2015). The ATG7 and ATG10 facilitate the amalgamation of ATG12 and ATG5 which recruits ATG16 resulting in the formation of ATG12-ATG5-ATG16 complex (ATG16L). ATG16L is utmost important for the process of sequestration which functions as E3 ubiquitin ligase (Fujita *et al.*, 2008). Microtubule-associated protein 1 light chain 3 (LC3) or also referred as ATG8, is cleaved by ATG4 to produce LC3-I. Attachment of LC3-I to phosphatidylethanolamine is driven by concerted actions of ATG7 E1-like enzyme and ATG3 E2-like enzyme. Subsequently, Atg16L E3 ubiquitin ligase promotes lipidation of PE-conjugated LC3-I to generate lipidated LC3-II which incorporates into the membrane of autophagosome, where it is recognized as a site of adaptor proteins for the cargo binding (Fujita *et al.*, 2008; Kabeya *et al.*, 2000). Engulfed cargo containing aggregated proteins and organelle substrates are ubiquitinated and recognized by autophagic adaptors such as p62/SQSTM1 which then delivered to autophagosome via binding to LC3 interacting domains (Matsumoto *et al.*, 2011; Rogov *et al.*, 2014). Furthermore, autophagosomes fuse with lysosomes to form autophagolysosomes prior to the process of degradation by lysosomal hydrolases concomitantly with the release of small macromolecules to cytosol by lysosomal permease (Fulda & Kogel, 2015; Kroemer & Jaattela, 2005; Mizushima, 2007). It has been proposed that exploitation of autophagy may serve as a novel therapeutic strategy for increasing susceptibility of cancer cells to chemotherapeutic agents. Mounting evidence revealed the double-edged sword properties of autophagy by either promoting cancer cell survival and tumourigenesis or inhibiting the cytoprotective effect resulted in cell death (Li *et al.*, 2013; Tsujimoto & Shimizu, 2005).

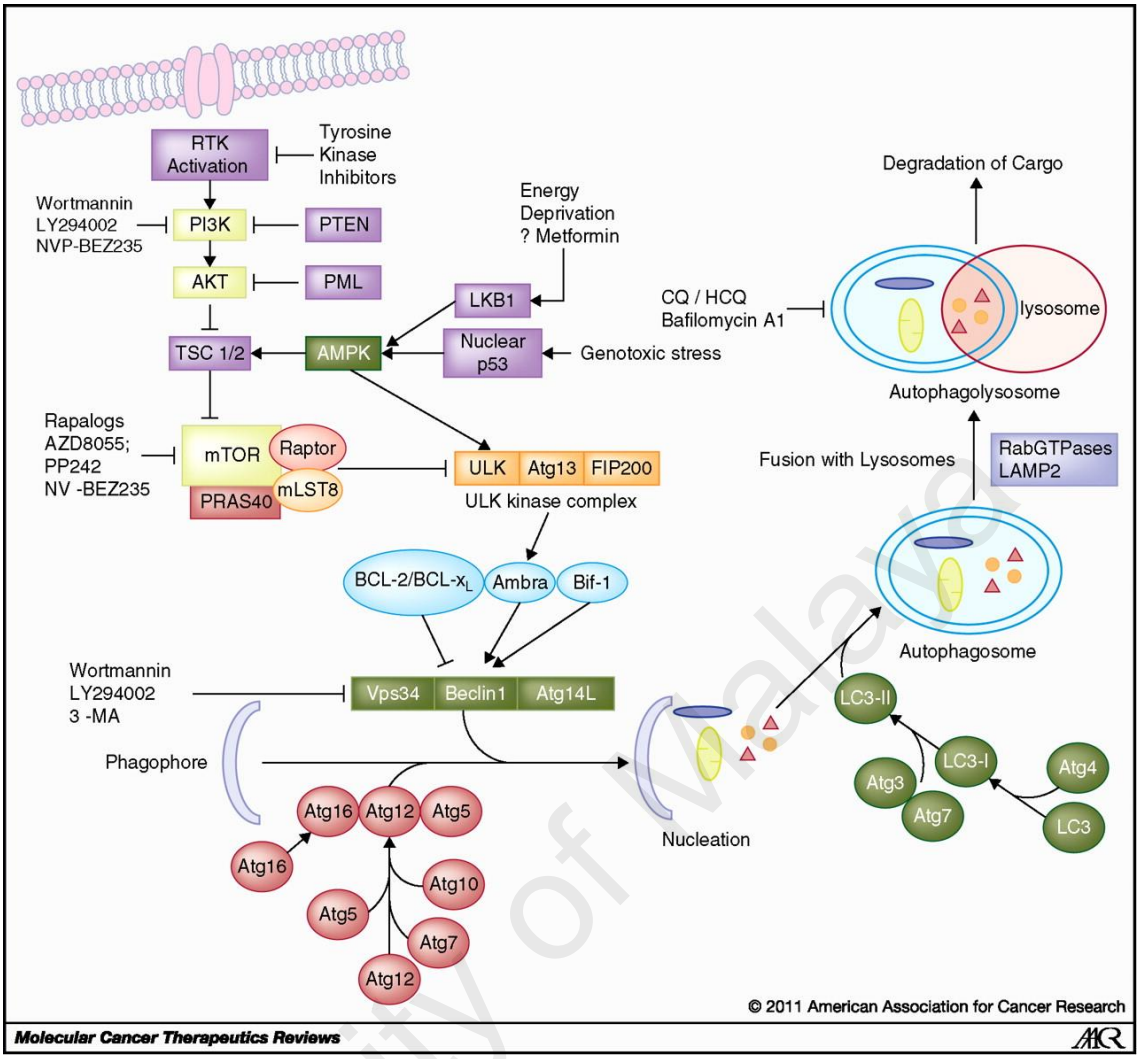

Figure 2.4: Autophagy pathway. AMPK, adenosine monophosphate kinase; ULK, UNC-51-like kinase. Adapted from Yang *et al.* (2011).

2.1.7 Cell cycle

Cell cycle, a conserved process which is required for cell proliferation and development. The progression of tumourigenesis is attributable to the defect in cell cycle checkpoint control resulting in aberrant dysregulation of cell cycle (Williams & Stoeber, 2012). Cell cycle subdivided into several phases comprising G_0 (Gap 0), G_1, S (synthesis), G_2 and M (mitosis) which involves growth, chromosomal replication and mitosis that are essential for cell division and replication. G0 is referred as quiescent which is in a resting state whereas for G1, S, G2 and M are referred as interphase. Upon stimulation of growth factors and nutrients, cells progress to enter G1 phase. Subsequently, mitogen stimulation releases the brakes of cell cycle progression allowing the transition of G1 to S phase to commit DNA synthesis by passing G1/S restriction checkpoint. Once DNA replication is completed, cells are progressed from S phase to G2 phase in which the fidelity is monitored and prepared for the entry into the M phase. During the M phase, two genomically stable daughter cells (cytokinesis) are produced from the division of the chromosomes and cytoplasm of cells (Dominguez *et al.*, 2015).

Cell cycle checkpoints are activated to induce cell cycle arrest further allowing the repair upon detection of defects during DNA synthesis or mitosis (Hartwell & Weinert, 1989; Malumbres & Barbacid, 2009). In addition, cell cycle checkpoints permit the initiation of various mechanisms including senescence, mitotic catastrophe and apoptosis to hinder propagation of severely damaged daughter cells. Growing evidence revealed that there are several checkpoints available: 1) G1/S checkpoint blocks cells with damaged DNA from entering S phase, 2) intra-S checkpoint interferes the replication of DNA during S phase in order to reduce the replication errors, and 3) G2/M checkpoint prevents the cells with faulty chromosome segregation from progressing to the phase of

mitosis. Attenuation of cell cycle checkpoints eventually promote the aberrant proliferation of peculiar cells leading to a diverse of diseases including cancer (Dominguez *et al.*, 2015; Viallard *et al.*, 2001). Thus, all these checkpoints are utmost important in minimizing defective genomics during cell cycle progression.

2.1.7.1 Cell cycle regulation

Cell cycle progression requires a concerted action of heterodimeric cyclin-dependent kinases complexes. Cyclin-Dependent Kinases (CDK) belongs to a family of serine/threonine protein kinases which consist of catalytic subunit CDK and activating subunit cyclins. Constitutive activation of CDK is usually dependent on the binding of respective cyclins in which the CDK-cyclin complexes regulates the cell cycle progression from one phase to another as shown in Figure 2.5.

Stimulation of mitogenic signalling such as RAS/RAF/MAPK pathways initiates the entry of G1 phase which inactivates ubiquitin ligase and anaphase-promoting complex/cyclosome complex by CDH1 phosphorylation (APC/C-CDH1) coupling with the activation of CDK4/6-cyclin D complexes. The activation of CDK4/6-cyclin D complexes is triggered through the binding of D-type cyclins consisting of D1, D2 and D3 on either CDK4 or CDK6. Consequently, CDK4/6-cyclin D complexes phosphorylate and inactivate tumour suppressor retinoblastoma (RB) proteins to allow the activation of E2F via transcription. This further permits the transcription of E types of cyclin (E1 and E2) as well as A types of cyclin which allosterically bind to CDK2 and surpassing restriction point (Ortega *et al.*, 2002). Both of these CDK2-cyclin E and CDK2-cyclin A are key regulatory complexes which is prerequisite for the entry of S-phase and mediate the initiation of DNA and centrosome duplication (Asghar *et al.*, 2015; Bassermann *et al.*, 2014). In contrast to CDK4/6, CDK2 is governed by CDK-interacting protein/kinase inhibitory protein (CIP/KIP) class of CDK inhibitors which interacts and inhibits CDK2-

cyclin complexes (Sherr & Roberts, 1999). For example, p21CIP1, an essential downstream target of p53 that attenuates DNA synthesis and p27KIP1 prevents the deregulated cell proliferation in response to mitogenic signalling (Polyak *et al.*, 1994; van den Heuvel & Harlow, 1993). At the end of S phase, CDK1 complexed with cyclin A facilitates the formation of RB-E2F complexes. Next, degradation of cyclin A-CDK1 by ubiquitin-mediated proteolysis results in the transition from S to G2 phase. In G2 phase, $SCF^{\beta\text{-}TrCP}$ promotes activation of CDK1 by triggering the degradation of CDK1 inhibitory kinase, Wee1 (Watanabe *et al.*, 2004). Upon entry to mitosis, CDK1 further complexed with type B of cyclins which are chief complexes to induce mitosis. Later, inactivation of CDK1-cyclin B complexes contributes to exit mitosis in which the CDK1-cyclin B complexes are degraded by the APC/C to facilitate the separation of chromosome and completion of mitosis as well as cytokinesis (Gavet & Pines, 2010).

2.1.8 Signalling pathways as anticancer molecular targets

2.1.8.1 PI3K/Akt/mTOR signalling pathway

Phosphatidyl-inositol-3-kinases/Akt and the mammalian target of rapamycin (PI3K/Akt/mTOR) signalling pathway plays an imperative role in various fundamental cellular processes including cell growth, apoptosis, proliferation, cell cycle progression and survival (Arcaro & Guerreiro, 2007). Aberrant activation of PI3K/Akt pathway dampens apoptotic response resulting in amplification of oncogenic signals and driving towards tumour progression (Hennessy *et al.*, 2005; Thorpe *et al.*, 2015). Considerable evidence has shown that PI3K/Akt/mTOR and NF-κB signalling is deregulated in numerous cancer cells particularly colorectal cancer whereby it enhances cancer progression and drug resistance (Danielsen *et al.*, 2015; Papadatos *et al.*, 2015). Thus, efforts have been made to develop therapeutic agents to target cancer and chemo resistance via this signalling pathway.

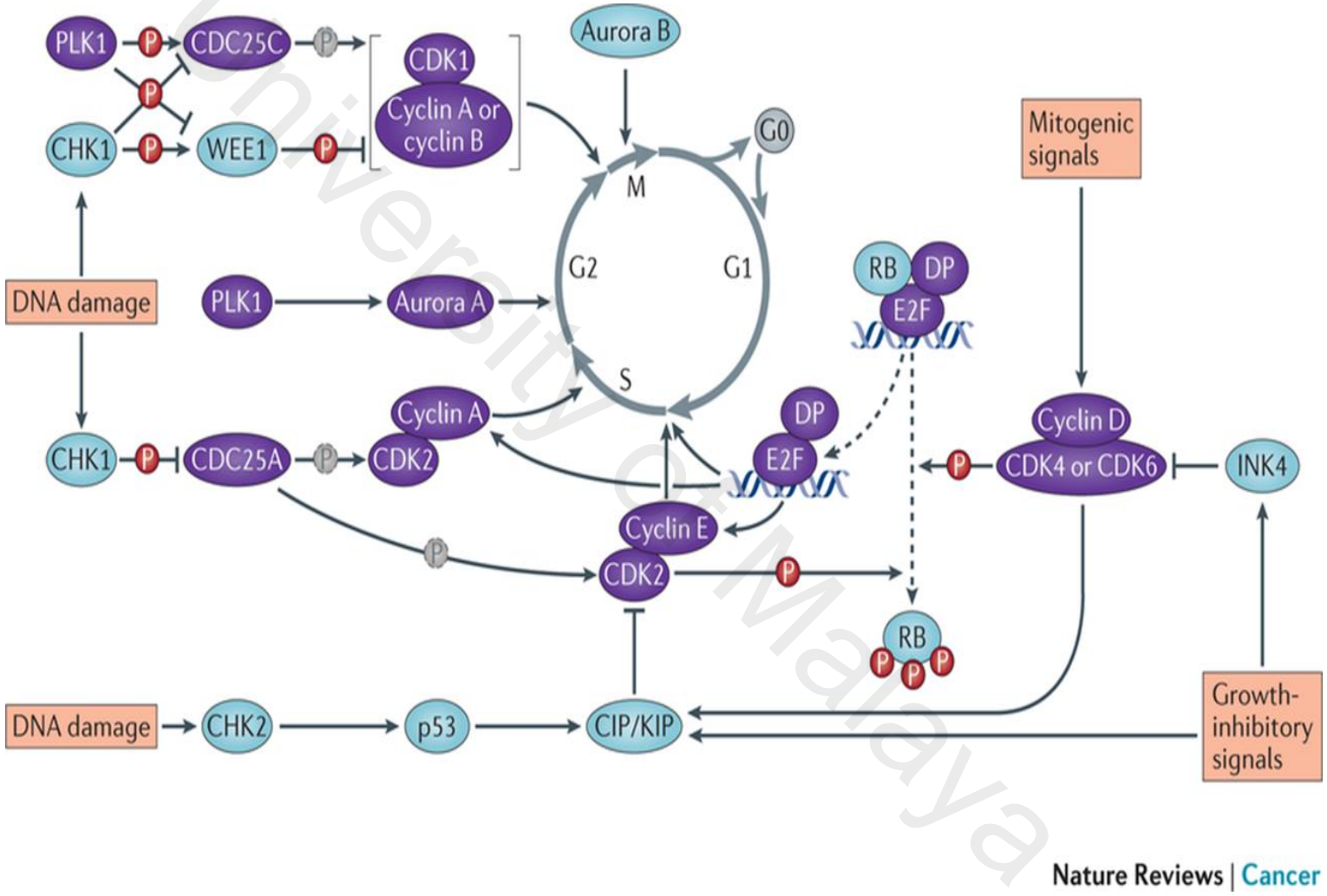

Figure 2.5: The involvement of several key regulatory proteins in different phases of cell cycle. Adapted from Otto and Sicinski (2017).

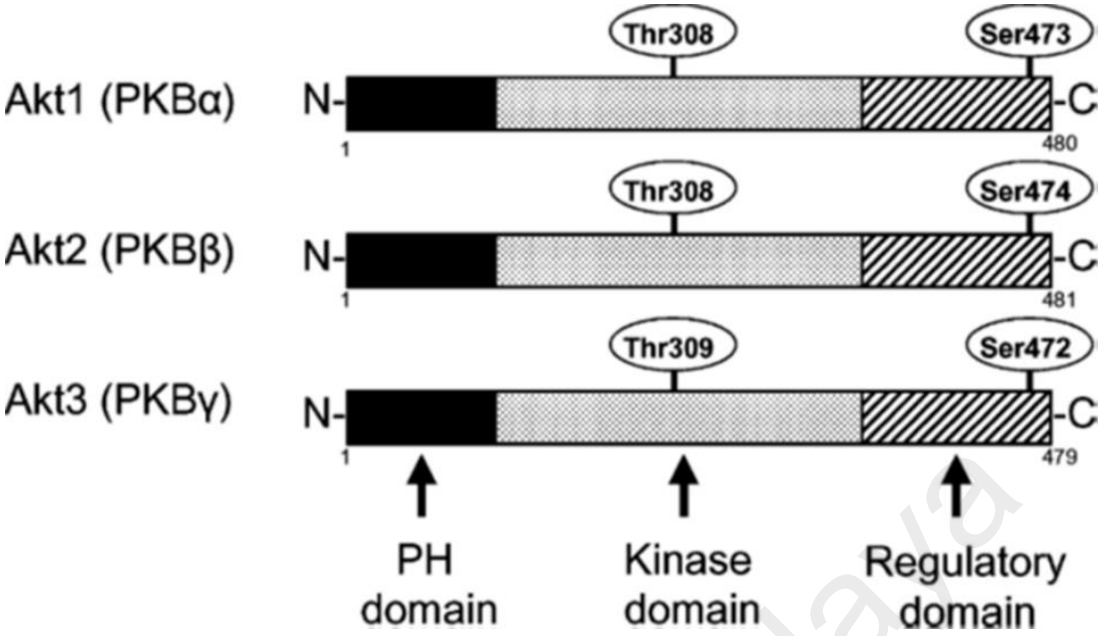

Figure 2.6: Isoform members of Akt/PKB. Adapted from Osaki *et al.* (2004).

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PI3Ks belong to lipid kinases family which are mainly categorised into three classes: (1) class I PI3Ks are heterodimers composed of a regulatory and a catalytic subunit, (2) class II PI3Ks are composed only with a catalytic subunit, and (3) class III are composed of a single member, Vps34 catalytic subunit (Cantley, 2002; Engelman *et al.*, 2006; Katso *et al.*, 2001). Among the classes, class I PI3Ks has been most widely studied (Carracedo & Pandolfi, 2008). Class I PI3K members are sub-classified into IA which can be stimulated by receptor tyrosine kinases (RTKs), and IB which can be stimulated by G-protein-coupled receptors (Engelman *et al.*, 2006). Class IA PI3Ks comprise of p85α, p85β or p85γ regulatory and p110α, p110β or p110γ catalytic subunit (Mellor *et al.*, 2012) whereas class IB PI3Ks comprise of p101 or p87 regulatory and p110γ catalytic subunit (Okkenhaug & Vanhaesebroeck, 2003). For class II PI3Ks, it can be induced by various receptors including Ras, integrins, cytokine receptors and RTKs. Class II PI3Ks comprises of three isoforms: PI3K-C2α, PI3K-C2β and PI3K-C2γ (Falasca & Maffucci, 2012).

Binding of growth factors to the RTKs such as insulin-like growth factor 1 receptor (IGF-1R), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor and platelet-derived growth factor receptor triggers activation of PI3K via phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Hu *et al.*, 1992). PIP3 acts as a second messenger to activate Akt signalling pathway by recruiting and activating phosphoinositide-dependent kinase 1 (PDK1) via pleckstrin homology (PH) domains. Full activation of Akt (PKBα) requires phosphorylation at the residue site of threonine 308 (Thr308) and serine 473 (Ser473). Hence, full activation of Akt preceded by first, PDK1 phosphorylates Akt at site of threonine 308 leading to the phosphorylation at serine 473 by mTORC2 (the complex rictor/mTOR) (Sarbassov *et al.*, 2005; Stokoe *et al.*, 1997).

Generally, human Akt family consists of three isoforms such as Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Osaki *et al.*, 2004). The structural of isoforms Akt is shown in Figure 2.6. Akt acts as a cardinal signalling nodes for PI3K/Akt pathway via modulating a diverse of intricate signalling pathways to promote cell cycle, cell survival, metabolism and angiogenesis (Stratikopoulos & Parsons, 2016). Activated Akt promotes cell survival, proliferation and cell cycle via phosphorylation of the downstream effectors including forkhead box O (FOXO), glycogen synthase kinase 3, BAD, p53 and p27 (Figure 2.7) (Manning & Cantley, 2007). Apart from these, Akt also activates mTOR complex 1 (mTORC1) at Ser2448 via phosphorylation of TSC2 which eventually activate S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) to promote protein synthesis (Ma & Blenis, 2009).

2.1.8.2 Mammalian target of rapamycin (mTOR)

The mTOR is regarded as an essential regulator of various fundamental processes including cell growth and survival. Growing attention has drawn recently of its involvement in the development of tumour. Upon stimulation of growth factors and nutrients, constitutive activation of mTOR along with the dysfunction of tumour suppressors results in tumour growth, angiogenesis and eventually metastasis (Faivre *et al.*, 2006). mTOR, a catalytic subunit can be distinguished into two functionally different complexes, mTOR Complex 1 (mTORC1) and 2 (mTORC2). Both of them share similar components which are mTOR, mLST8 and deptor. As shown in Figure 2.8, specifically for mTORC1, it composed of raptor and PRAS40 whereas for mTORC2 composed of rictor, mSin1 and Protor (Zoncu *et al.*, 2011). mTORC1 which is sensitive to rapamycin has shown to involve in PI3K/Akt pathway. It is a downstream molecule of AKT whereby Akt activates mTORC1 by attenuating tuberous sclerosis protein 2 (TSC2) and PRAS40 through phosphorylation. Thus, it leads to the activation of S6K1 and inactivation of

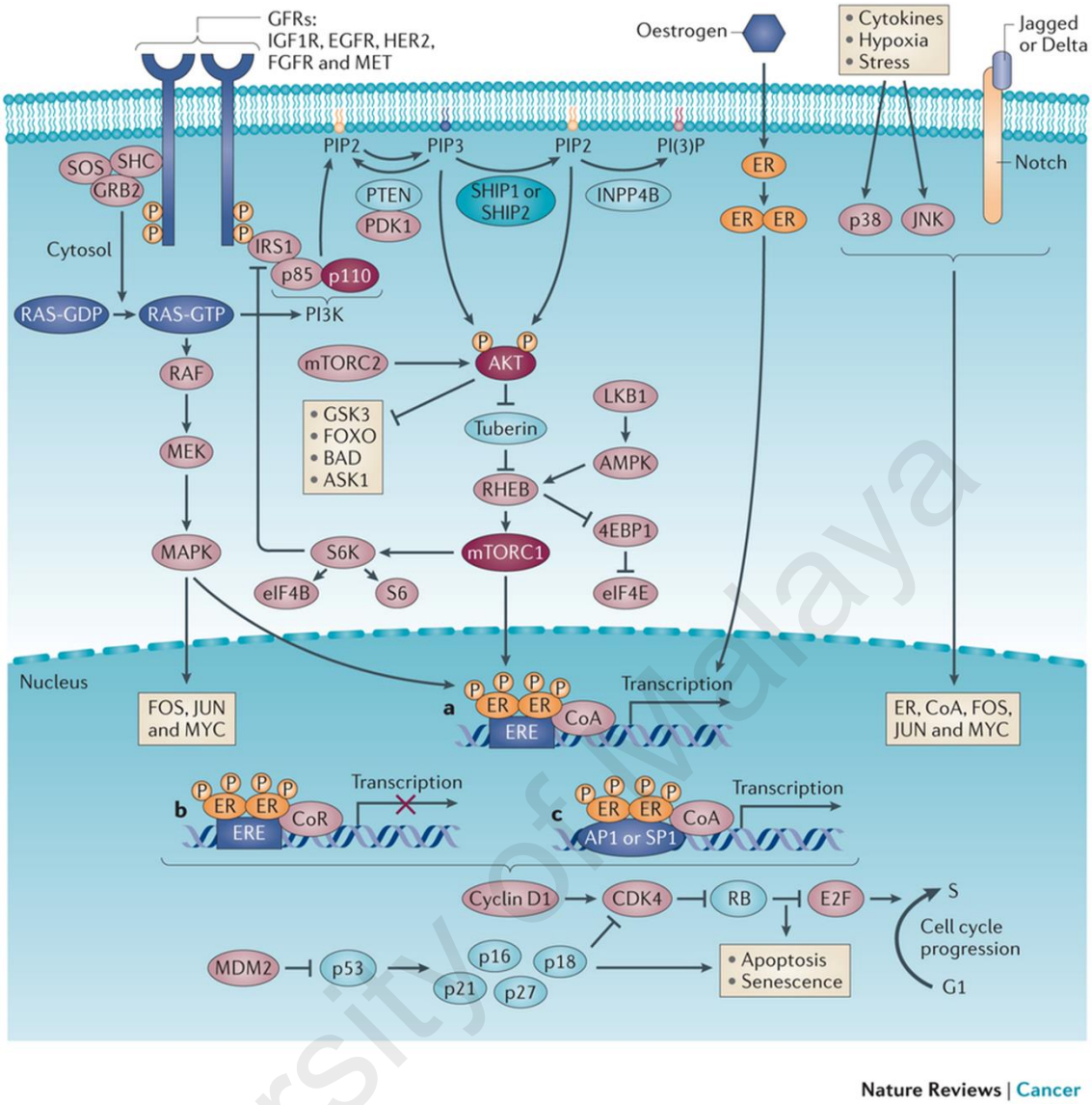


Figure 2.7: Regulatory PI3K/Akt/mTOR signalling pathway couples with inactivation of PTEN which lead to various cellular processes. Adapted from Ma *et al.* (2015).

4EBP1 in order to promote protein synthesis (Hay & Sonenberg, 2004). In contrast, mTORC2 is considered to be less sensitive to rapamycin, which act as an upstream mediators of Akt. It is activated upon exposure of growth factors to allow the full activation of Akt by phosphorylating Akt at the residue site of Ser473 (Sarbassov *et al.*, 2005).

2.1.8.3 Phosphatase and tensin homolog deleted on chromosome 10 (PTEN)

Over constitutive activation of PI3K/Akt and mutation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) are often identified in various malignancy including prostate, breast and colorectal cancer (Li *et al.*, 1997; Molinari & Frattini, 2013). PTEN is a dual lipid and protein phosphatase which has emerged as a tumour suppressor. PTEN is constitutively orchestrated by numerous factors such as early growth regulated transcription factor 1, insulin-like growth factor 2, transforming growth factor β and p53 (Li & Sun, 1997; Moorehead *et al.*, 2003; Stambolic *et al.*, 2001; Virolle *et al.*, 2001). It negatively orchestrates the PI3K/Akt pathway by converting PIP3 to PIP2 via dephosphorylation and thus antagonizing the function of PI3K. In contrast, mutation or loss of PTEN leading to persistent activation of PI3K/Akt and excessive accumulation of PIP3 contribute in cancer progression by enhancing cell proliferation, survival, apoptosis resistance, angiogenesis, cell migration and metastasis (Zhang & Yu, 2010).

2.1.8.4 MAPK pathway

Mitogen-activated protein kinases (MAPKs) are conserved serine-threonine kinases that contribute in a multitude of functional cellular processes including survival, cell proliferation, apoptosis, differentiation and transformation (Dhillon *et al.*, 2007; McCubrey *et al.*, 2006). It was established that aberrant regulation of MAPK signalling has been correlated in various physio pathological conditions including cancers,

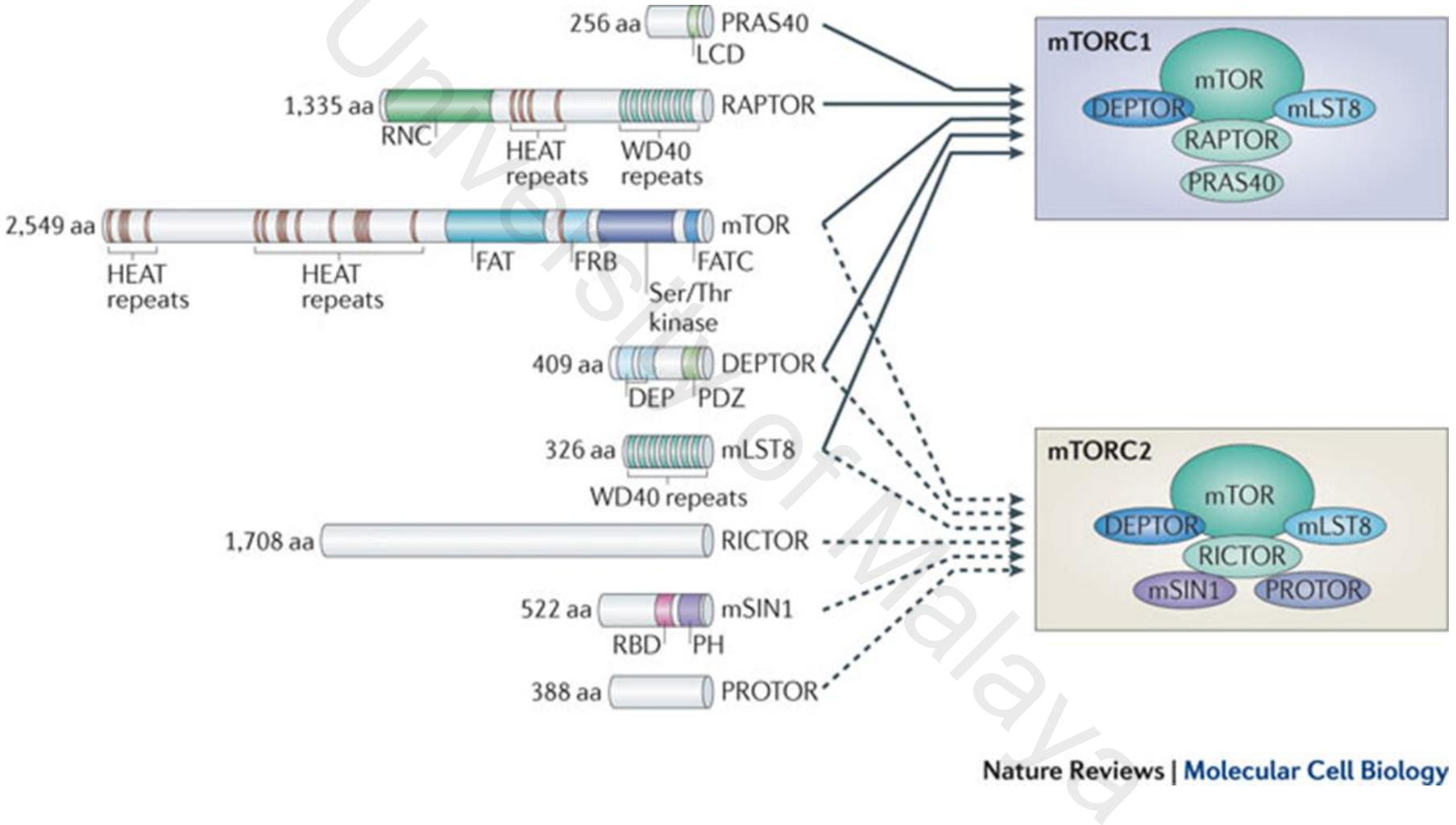


Figure 2.8: Structural domain of mTOR and the interacting proteins. Adapted from Zoncu *et al.* (2011).

Alzheimer's disease (AD), Parkinson's disease and diabetes (Kim & Choi, 2010; Lawrence *et al.*, 2008). A number of mammalian MAPK family members has been discovered, however only few distinct groups are widely studied which including extracellular signal-regulated kinase 1 to 8 (ERK1-8), p38 MAPK α, β, γ and δ (p38 α-δ), c-Jun NH2-terminal kinase 1 to 3 (JNK1-3; or stress-activated protein kinase/SAPK) and ERK5 (Kim & Choi, 2010; Wortzel & Seger, 2011). In contrast, ERK3, ERK4, ERK7/8 and NLK are the less characterized MAPK family members. Each of their respective structure and domains are illustrated in Figure 2.9. MAPKs cascade transduces in response to a plethora of extracellular signalling stimuli: ERK1/2 is triggered by growth stimuli such as platelet-derived growth factor, while JNKs and p38 MAPK are activated upon stimulation of inflammatory cytokines, DNA damage and numerous cellular stress such as oxidative stress and endoplasmic reticulum stress (Kim & Choi, 2010; Seger & Krebs, 1995).

Activation of MAPKs involves a series of phosphorylation cascades by first interacting with diverse receptors including receptor tyrosine kinase (RTKs) and G protein-coupled receptors in response to trigger of extracellular stimuli. These lead to the binding of small GTP-binding protein of the Ras/Rho family to activate MAPK kinase kinase (MAPKKK) (Dan *et al.*, 2001; Kolch, 2000). Consequently, activation of MAPKKKs transmits signal to facilitate the phosphorylation of MAPKK followed by phosphorylation on MAPKs (Roux & Blenis, 2004). MAPKs primarily activate a multitude of downstream mediators which in turn mediate array of cellular processes such as mitosis, embryogenesis, cell differentiation, movement, metabolism and programmed death (Chen *et al.*, 2001; Platanias, 2003).

ERK1/2 is identified to possess a double edged sword functions which are anti-apoptotic or pro-apoptotic functions. Generally, ERK1/2 is initiated by a cascade of

phosphorylation via a small G protein Ras-Raf family member followed by activation of MEK1/2 and ERK1/2. ERK transcribes several transcription factors such as ternary complex factor Elk-1, serum response factor accessory protein Sap-1a, Ets1, p53, c-Jun, c-Fos, and c-Myc (Zhang & Liu, 2002) integrating cell survival signal. However, prolonged activation of ERK1/2 may confer pro-apoptotic functions in various diseases which has been reported in several studies. Persistent activation of ERK1/2 is found to cause oxidative toxicity towards glutamate-induced neuronal cells (Stanciu *et al.*, 2000; Subramaniam *et al.*, 2004). Besides, suppression of ERK1/2 has been revealed to develop drug resistance in several cancer cell lines and decreasing the apoptosis induction resulting in cancer progression (Yeh *et al.*, 2004; Yeh *et al.*, 2002). Numerous natural-derived phytochemicals was discovered to activate ERK1/2 causing cell death via cell cycle arrest and promoting apoptosis in cancer (Chen *et al.*, 2016; Nguyen *et al.*, 2008; Tong *et al.*, 2011; Wu *et al.*, 2014).

JNK exists in several isoforms encompassing JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously found in almost every cells while JNK3 is mainly found in the brain and heart tissues (Chang & Karin, 2001; Davis, 2000). For p38 MAPK protein, p38α, p38β and p38δ are identified, however only p38α is well characterized. For both p38 and JNK signalling pathway is activated via MAPKKKs including ASK1, TAK1, MEKK1 or MLK3 which then followed by the activation of MAPKK where JNK is transduced by MKK4 or MKK7 and p38 is transduced by MKK3 or MKK6 in response to cytokines, stress and DNA damage stimuli (Lin, 2003). This results in activation of various transcription factors such as p53, Bax, CREB, c-Jun, activating transcription factor 2 and c-Myc which depicted in Figure 2.11 (Wagner & Nebreda, 2009).

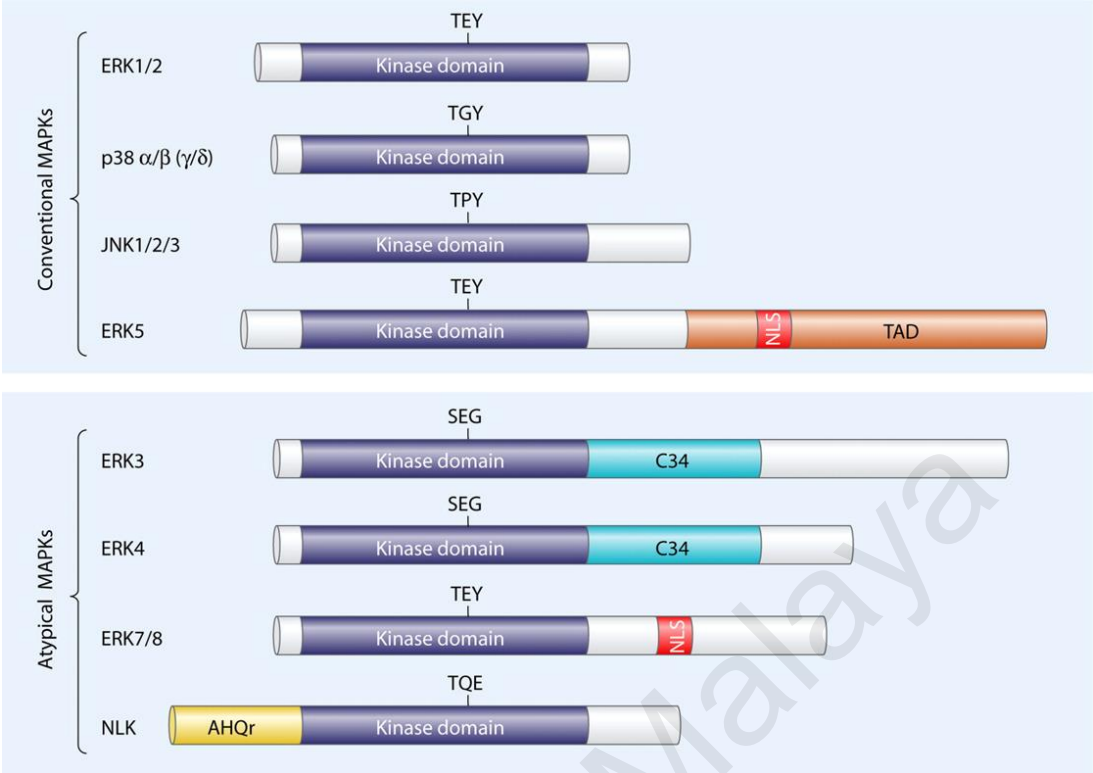


Figure 2.9: Functional structure and domain of MAPKs subfamilies. Adapted from Cargnello and Roux (2011).

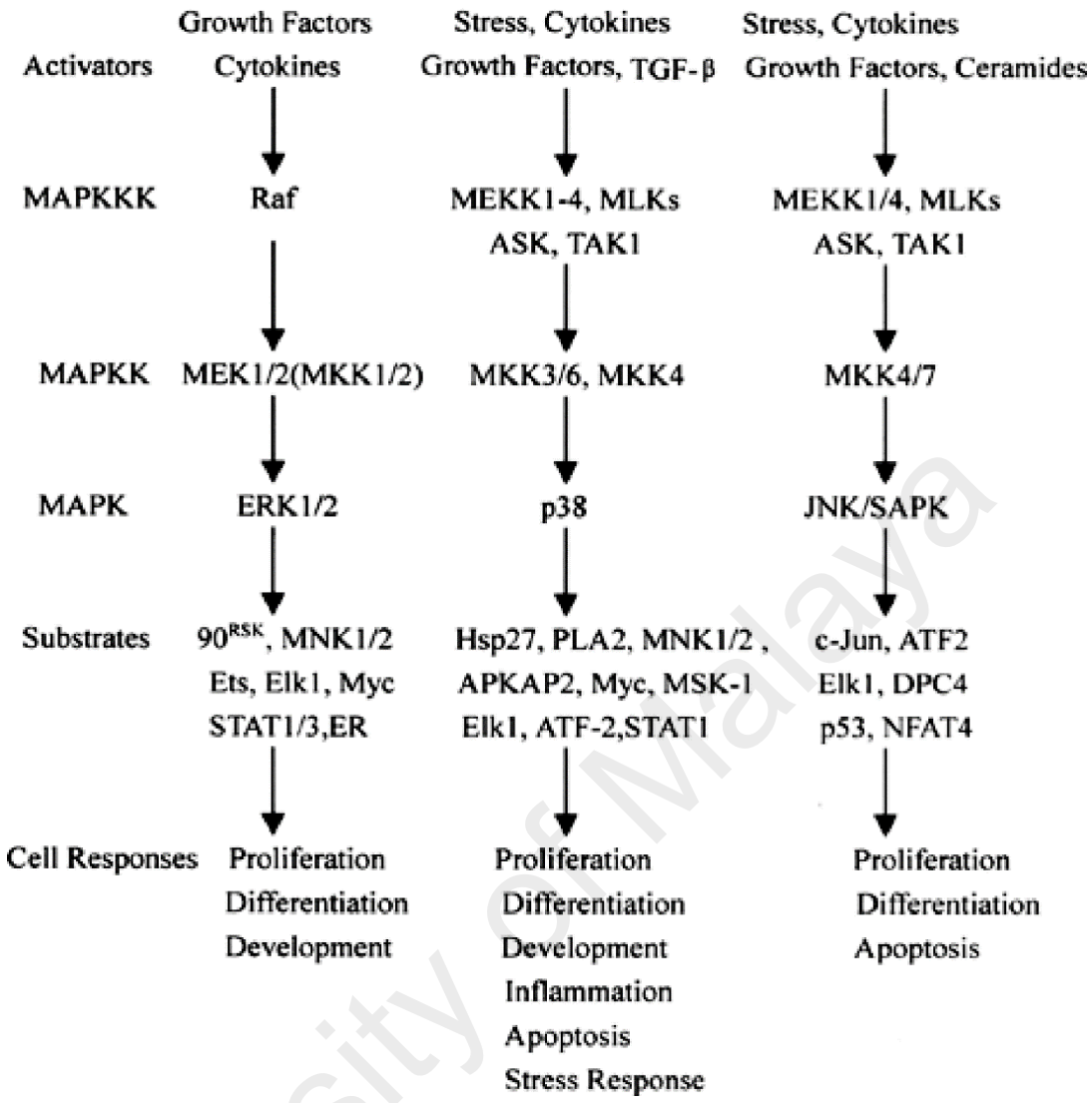


Figure 2.10: Activation of MAPKs signalling leads to multiple cellular responses. Adapted from Zhang and Liu (2002).

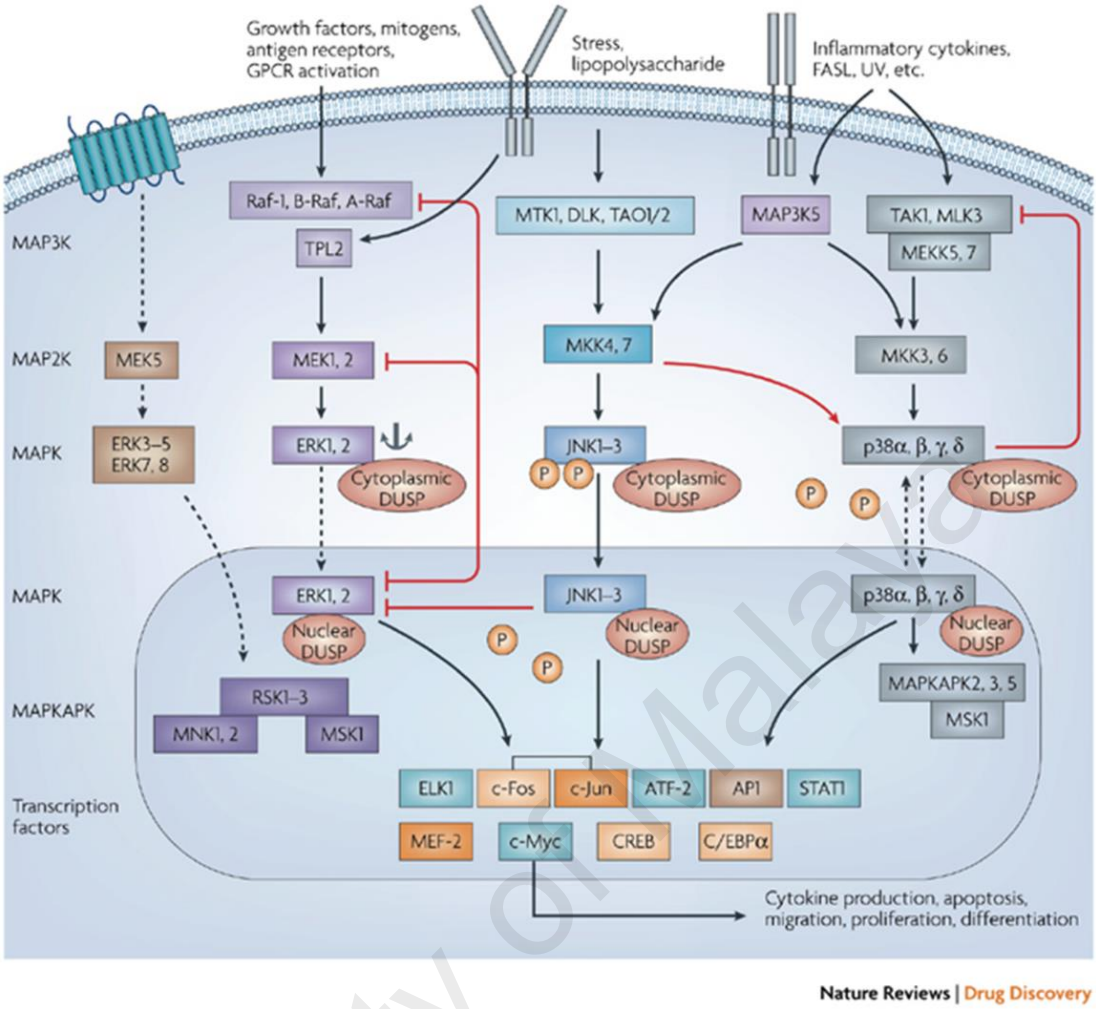


Figure 2.11: Detailed mechanisms of each of the subfamilies of MAPKs signalling pathway. Adapted from Jeffrey *et al.* (2007).

2.1.9 Oxidative stress

Oxidative stress is a result of the perturbation in the balance where oxidant is favoured over antioxidant. This condition also can be defined as excessive release of reactive oxygen species that is beyond the ability of antioxidants to counteract. Oxidative stress is implicated in various pathogenesis including cancer, neurodegenerative diseases, aging and diabetes (Birben *et al.*, 2012). Reactive oxygen/nitrogen species can be represented by free radicals which comprised superoxide anion radical, alkoxyl, hydroxyl, lipid peroxyl radicals, nitric oxide and peroxynitrite, and non-free radicals such as hydrogen peroxide, ozone, singlet oxygen, hypochlorous acid, nitrous acid and lipid peroxide (Genestra, 2007). Different degrees of oxidative stress exerts and dictates different effects on biological responses. The presence of low or moderate concentrations of ROS/RNS bring about several outcomes including sustained cell proliferation and differentiation leading to stress-responsive survival pathway (Janssen *et al.*, 2008). In contrast, high levels of ROS/RNS exert deleterious effects which results in the damage of cellular components such as DNA, lipid, protein and membranes (Gorrini *et al.*, 2013). Among the components, lipid is the most prevalent and susceptible to attacks by ROS. The free radicals damage lipids via lipid peroxidation causing a detrimental effect against the cell membranes. This oxidative attack promotes the formation of reactive aldehydes such as malondialdehyde, 4-hydroxy-2-nonenal and conjugated diene compounds (Valko *et al.*, 2007). The aldehydes can diffuse within or away from the original sites which amplify cellular damage. Consequently, these can alter the biological responses of the membrane and inactivate membrane-bound receptors leading to impaired cellular functions (Kurutas, 2016).

Free radicals are able to destroy the purine and pyrimidine bases as well the deoxyribose backbone of DNA molecule. These modifications of genetic materials

arising from oxidative attack initiates mutagenesis, tumourigenesis and ageing (Halliwell, 2007; Valko *et al.*, 2007). For proteins, peptides or amino acids exposed to oxidative stress, the side chains of amino acids particularly cysteine and methionine residues were most susceptible to the attack by ROS/RNS to undergo oxidation (Stadtman, 2004). Oxidation by ROS/RNS may lead to conformational alterations, protein unfolding and fragmentation of peptides thus protein activities and biological functions were interrupted (Birben *et al.*, 2012; Dean *et al.*, 1985).

2.1.9.1 Oxidative stress and cancer

In cancer, oxidative stress acts as prominent causative factor in the development of cancer as well as curative purpose. However, increasing evidence report that ROS possesses both oncogenic as well as therapeutic effects. Under different circumstances, different ROS levels may initiate diverse of biological responses. At low to moderate levels, ROS functions as a positive regulator either by propagating the signal favouring cellular proliferation and differentiation in normal cells. In response to the moderate stimulation of oxidative stress ROS, several pathways can be activated, for instance cell cycle, JNK, MAPK and ERK which are associated with the cell growth and survival in cancer cells (Dhillon *et al.*, 2007; Gorrini *et al.*, 2013). P53 is known as guardian of genome or tumour suppressor owing to its prominent role in preventing DNA-damaged cells from dividing (Fischer, 2017; Vogelstein *et al.*, 2000). However, p53 is mutated by direct action of ROS thereby preventing apoptosis and promoting cancer progression as well as DNA damage (Miller & Koeffler, 1993). Copious studies have reported that more than 80% of the mutation on p53 was found in human cancers suggesting that this deficiency promotes the formation and progression of cancer (Olivier *et al.*, 2010; Rivlin *et al.*, 2011). High intracellular ROS levels is a driving force of the tumour formation and a new redox balance is composed in cancer cells to neutralize the excessive of ROS

production leading to cellular adaptation and proliferation (Gorrini *et al.*, 2013; Sosa *et al.*, 2013). As mentioned above, despite oxidative stress arising from the stimulation by ROS to promote cancer cell survival and proliferation, accumulation of ROS can also improve the sensitivity of cancer cells towards treatment. Ample evidences has unravelled that modulation of oxidative stress can be an essential therapeutic approach, in which increasing ROS to reach toxicity threshold thereby render cancer cells more susceptible to the onset of apoptosis conferred by the ROS-inducing chemotherapeutic drugs (Galadari *et al.*, 2017; Trachootham *et al.*, 2009). Excessive ROS can be tumour suppressive, hence, results in a diverse cell death and potential cellular damage which act as a pro-oxidant therapy in cancer (Gorrini *et al.*, 2013; Simon *et al.*, 2000).

Aberrant generation of ROS may activate several stress-inducible signalling pathways such as NF-κB, MAPK pathway and autophagy which eventually converges on the transduction of apoptotic signals (Davis, 2000; Karin *et al.*, 2002; Tsujimoto & Shimizu, 2005). Notably, stimulation of extracellular signals including oxidative stress, mitogen-activated protein kinase (MAPK) signalling pathways mediate intrinsic signalling involved in various cellular events including cell proliferation, survival, differentiation, growth, autophagy and apoptosis. Stress-activated c-Jun NH2-terminal kinase (JNK), the extracellular signal-regulated protein kinases (ERK1/2, p44/p42) and p38-MAPK are regarded as three main members of MAPK family which participate in kinase cascade (Dhillon *et al.*, 2007). It is well documented that excessive ROS instigates the onset of MAPK signalling transduction via the integral ERK, JNK and p38 MAPK which is sequentially followed by induction of apoptosis (McCubrey *et al.*, 2006).

To date, numerous anticancer drugs or natural derived compounds have been reported to act as pro-oxidant therapy candidates to eradicate the bulk of cancer cells. However, the challenge remains to categorize cancer types that may benefit from pro-oxidant

therapy. Currently, various cancer therapeutic drugs amplify ROS production to reach a threshold which lead to cancer cell lethality or susceptibility towards therapies (Harris & Brugge, 2015).

2.2 Neuroinflammation and Neurodegenerative disease

The human central nervous system (CNS) is paramount important in regulating all the organs in human body through a network of neurons and glia cells including astrocytes, microglia and oligodendrocytes (Azevedo *et al.*, 2009). Decades ago, CNS was frequently regarded as immune privileged which tightly restricts the entry of nutrients and peripheral immune cells. Hence, this consequently limits the communication between immune activation and CNS pertaining to the presence of blood brain barriers (Carson *et al.*, 2006). However, current perception has clearly accentuated the existence of communication between the peripheral immune system and the CNS. Both innate and adaptive inflammatory events do emerge in CNS as well as infiltration of peripheral immune cells apart from direct infections or injuries which are termed as neuroinflammation (Amor *et al.*, 2010; Fischer & Maier, 2015; Wee, 2010). Inflammatory response is a protective mechanism to recuperate, regenerate and remove the damaged cells or infectious agents.

In CNS, the onset of neuroinflammation is coordinated by immunocompetent cells, microglia (the main effector of innate immunity) and astrocytes (Bernhardi, 2007). Under normal physiological conditions, microglia remain in inactive state which is characterized by ramified morphology (Hanisch & Kettenmann, 2007). Microglia cells, the resident macrophages of CNS, are prime components of intrinsic CNS immune system which serve as immune surveillance and first line of host defence to restore and repair the

damaged glia or neuronal cells (Shabab *et al.*, 2017; Streit & Xue, 2010). However, prolonged stimulation of inflammatory signals could result in deleterious effects in which the microglia are overactivated (microgliosis) leading to permanent neuronal damage and defect in regeneration (Figure 2.12) (Chen, 2016; Russo & McGavern, 2016). Neurodegenerative diseases are manifested by various common pathological characteristics including aberrant protein aggregation (Ross & Poirier, 2005), inflammation (Zipp & Aktas, 2006), oxidative stress (Andersen, 2004) and neuronal apoptosis (Craggs & Kalaria, 2011; Okouchi *et al.*, 2007). Epidemiological evidence has demonstrated that neuroinflammation is recognized as common causative factor to the pathogenesis of neurodegenerative diseases by triggering the damage on neuronal cells. Therefore, chronic neuroinflammation contributes to a variety of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and multiple sclerosis (Chen, 2016) which is regarded as one of the biggest global public health and social care challenges encountered by people today and possibly in the future. The event of neuroinflammation can be characterized by the excessive release of pro-inflammatory molecules such as ROS, cytokines, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) results in neuronal damage and degeneration (Rubio & Morillas, 2012). It is noteworthy that this aberrant generation of pro-inflammatory mediators appear to be an accessible link between the early deposition of amyloid-β and the phosphorylation of tau which leads to neuronal demise particularly in Alzheimer's disease (Birch *et al.*, 2014; Heneka *et al.*, 2014).

2.2.1 Oxidative stress and neurodegenerative diseases

Oxidative stress is one of the leading cause of inflammation which has been implicated in the neurodegenerative diseases especially AD (Markesbery, 1997; Valko *et al.*, 2007)**.** Perturbation in redox balance leads to neuronal damage and hence induces the initiation

of brain-inflammatory disorders including AD (Figure 2.13) (Shi & Gibson, 2007). It is well documented that the contribution of oxidative stress in the β- amyloid peptide (Aβ) deposition which further promotes the aggregation of plaques in the brain leading to the development of AD in most patients (Butterfield *et al.*, 2002). ROS acting as secondary messenger was found to associate with the pathogenesis of AD, indicating oxidative stress is an early hallmark of AD. Excessive accumulation of ROS modulated the lipids, DNA, membranes and proteins leading to the interruption of protein folding, production of anti-inflammatory mediators and impairment of cellular functions which exacerbates the inflammatory responses including production of pro-inflammatory cytokines. Consequently, the oxidative stress-induced inflammatory signals activate glial cells. Impairment in antioxidant defence mechanism along with the oxidative stress, facilitated the vicious cycle to enhance the deposition of Aβ via positive feedback loop, forming amyloid plaques and eventually cellular demise in neuronal cells (Chan, 2001; Fulda *et al.*, 2010). Therefore, the balance between oxidative stress/inflammation and antioxidants/anti-inflammatory mediators is crucial in counteracting neuroinflammatory and neurodegenerative related diseases.

2.2.2 Antioxidants

Antioxidants is defined as a substance that delays, deters or eliminates oxidative stress to reduce destruction to a target molecule by neutralizing the free radicals (Halliwell, 2007). Antioxidant systems are of paramount importance as it acts as scavengers to counterbalance oxidative stress thus prevents or delays malignant transformation and development of neurodegenerative diseases (Valko *et al.*, 2006). Oxidative stress can be countered by either endogenous or exogenous antioxidants. Exogenous antioxidants are derived from sources such as fruits, vegetables or dietary supplements containing vitamin C, carotenoids, vitamin E, phenolic acids, cinnamic acid derivatives and flavonoids.

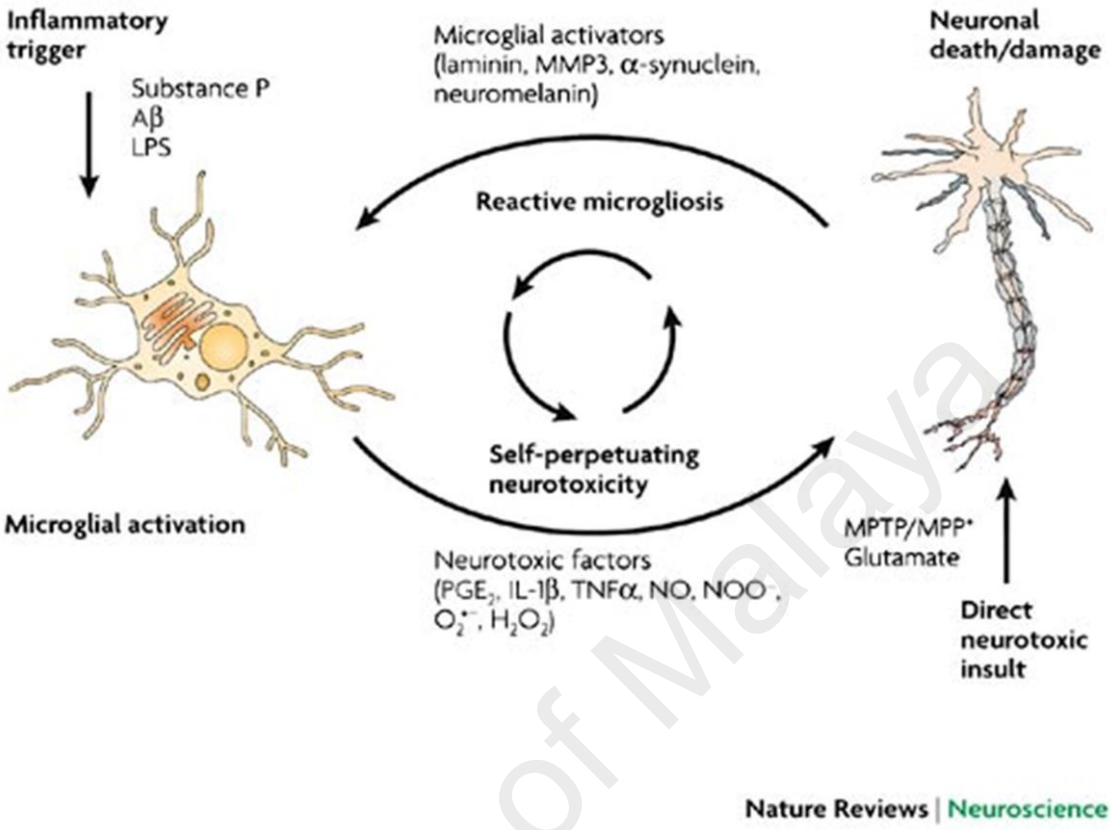


Figure 2.12: Microgliosis, a driving force of neuronal cell death in response of inflammation. Adapted from Block *et al.* (2007).

(Pisoschi & Pop, 2015). In contrast, endogenous antioxidants produced *in situ* in human body can be categorized into enzymatic and non-enzymatic antioxidants which are distributed in various compartments of the cells. Enzymatic antioxidants comprise superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, catalase (CAT) and heme oxygenase-1 (HO-1) whereas non- enzymatic antioxidants consist of enzymatic antioxidants consist of vitamin E, glutathione, vitamin c, carotenoids and flavonoids (Birben *et al.*, 2012; Pisoschi & Pop, 2015). Enzymatic antioxidants function by breaking down and eliminating free radicals. Generally, enzymatic antioxidants involve a multi-step process converting oxidants to hydrogen peroxide (H_2O_2) and subsequently to water in the presence of cofactors including copper, zinc, manganese and iron. For non-enzymatic antioxidants, it functions by interfering the free radical chain reactions (Nimse & Pal, 2015).

2.2.2.1 Superoxide dismutase (SOD)

SOD belongs to endogenous antioxidant enzyme which is commonly distributed in different cellular compartments. It exists in several isoforms including: 1) SOD1 (copper-zinc SOD) is found in the cytoplasm, nucleus and plasma membrane; 2) SOD2 (manganese SOD) is mainly found in mitochondria; and 3) SOD3 (copper-zinc SOD) is found in extracellular compartment. SOD functions as the first line defence system to remove ROS which scavenges superoxide radicals by catalysing the dismutation of $O^{2\bullet-}$ to H_2O_2 and O_2 as depicted in Figure 2.14 (Birben *et al.*, 2012; Lushchak, 2014; Sen & Chakraborty, 2011).

2.2.2.2 Catalase (CAT)

Catalase appears as a tetramer composed of four identical monomers with each of the monomer consisting of porphyrin heme group located at the active site. Degradation of

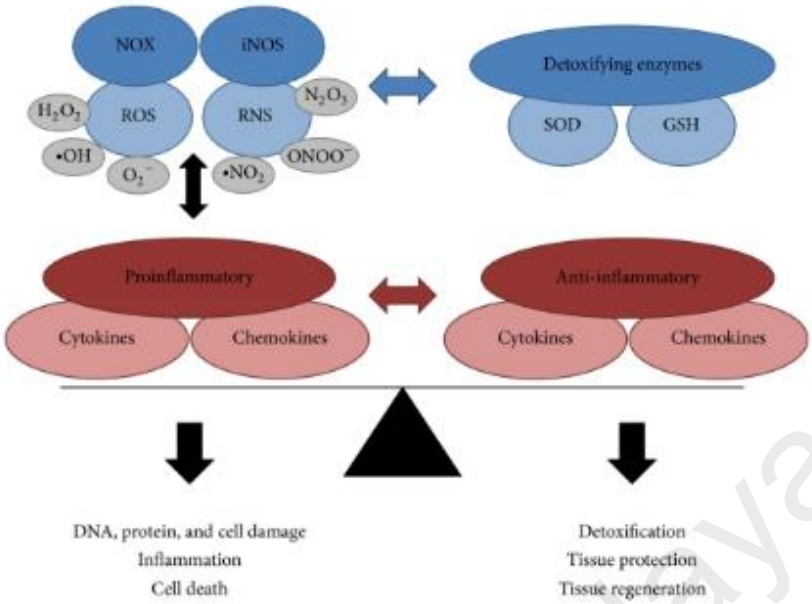


Figure 2.13: The equilibrium between antioxidants/anti-inflammatory mediators and pro-inflammatory mediators/oxidative stress. Adapted from Fischer and Maier (2015).

$$O^{2\bullet-} \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2 \quad (2)$$

Figure 2.14: Reaction of superoxide radicals catalysed by superoxide dismutase and catalase. Adapted and modified from Lushchak (2014).

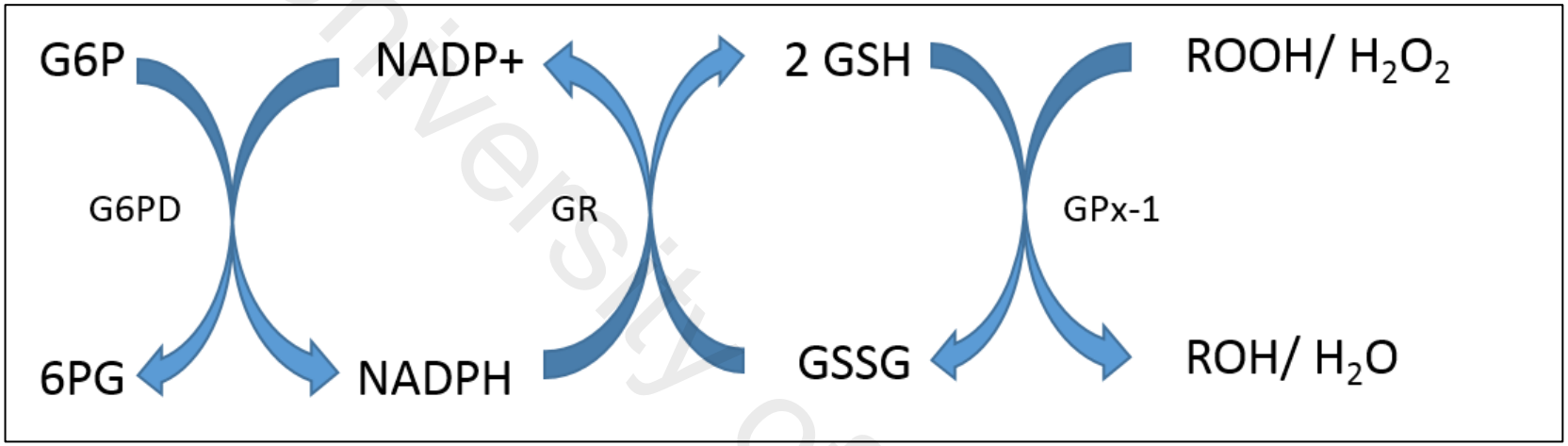


Figure 2.15: Detoxification of hydrogen peroxide and lipid hydroperoxides involving enzymes glutathione peroxidase and glutathione reductase. Adapted from Lubos *et al.* (2011).

H_2O_2 to water and oxygen, a process facilitated via interconversion between catalase-ferricatalase and compound I which is iron bound with an oxygen atom (Figure 2.14) (Birben *et al.*, 2012). Degradation of millions H_2O_2 molecules can be catalysed by each catalase in every second (Sen & Chakraborty, 2011).

2.2.2.3 Glutathione peroxidase (GPx)

Glutathione peroxidase, a tetrameric enzyme comprises one selenium atom at each monomer catalytic site and a selenocysteine at the active site (Day, 2009). Glutathione peroxidase exists in several isoforms including: GPx1, GPx2, GPx3 and GPx4 which catalyses the reaction between glutathione and H_2O_2 to produce water and oxidized glutathione (GSSG). Among these isoforms, GPx-1 is the most abundant enzyme which is localized in the cytosol and mitochondria of most mammalian cells (Lubos *et al.*, 2011). Besides, GPx1 is also regarded as a chief enzyme participating in detoxifying H_2O_2. Generally, GPx functions to prevent the accumulation of H_2O_2 and protects cells from oxidative stress caused by hydroxyl radical generated from H_2O_2. The removal of H_2O_2 by GPx into H_2O involves a number of secondary enzymes including glutathione reductase, glucose-6-phosphate dehydrogenase and cofactors (reduced glutathione, NADPH and glucose-6-phosphate) in which coupled with oxidation of GSH. Reduced glutathione donates electron in the process of oxidation whereby it is converted to glutathione disulphide (GSSG) as demonstrated in Figure 2.15 Subsequently, GSSG is reduced to two glutathiones coupled by glutathione Reductase in the presence of reducing equivalents nicotinamide adenine dinucleotide phosphate (NADPH). Simultaneously, glucose-6-phosphate dehydrogenase (G6PD) functions to maintain the supply of NADPH for the reaction (Lubos *et al.*, 2011).

2.2.3 Nrf2/HO-1 signalling pathway

Apart from all the antioxidant enzymes, phase II detoxification mechanism is another alternative defence system which is triggered under stress circumstances. This is orchestrated by nuclear factor erythroid 2-related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1). Transcription factor Nrf2 is a member of the cap'n'collar family of basic region-leucine zipper transcription factors which is ubiquitously distributed in the body as well as in CNS including astroglia, neurons and microglia (Moi *et al.*, 1994; Yamazaki *et al.*, 2015). Under normal physiological conditions, Nrf2 is usually sequestered in cytoplasm. The repressor protein Kelch-like ECH-associated protein-1 (Keap1) which is commonly anchored in the cytoplasm plays an important role in counteracting oxidative stress by tightly governing Nrf2 pathway (Kobayashi *et al.*, 2004). As illustrated in Figure 2.16, Keap1 complexes with an E3 ubiquitin ligase (Rbx-1) bind to cullin-3 further inhibits transcriptional activity of Nrf2. This complex drives the ubiquitination of Nrf2 and proteosomal degradation (Ahmed *et al.*, 2017; Cullinan *et al.*, 2004). The heterodimerisation of Nrf2 and Keap1 is via interaction between the Keap1 KELCH domain with the DLG as a latch and ETGE motifs as a hinge on Nrf2-Neh2 domain in the cytosol (Tong *et al.*, 2007). In contrast, the cellular Nrf2 is drastically enhanced in the presence of Nrf2 activators and oxidative stress stimuli. Oxidative stress stimulation results in conformational changes of Keap1 by modifying the reactive cysteine residues in Keap1 leading to dissociation of Nrf2 from Keap1. Keap1 is inactivated by these oxidative modifications and consequently stabilized Nrf2 by preventing the ubiquitination of Nrf2 and proteasomal degradation (Hayes *et al.*, 2010). The free Nrf2 translocates into the nucleus to heterodimerize with small Maf proteins. This complex binds and activates an antioxidant response element (ARE) battery of genes encoding antioxidative enzymes and detoxifying enzymes which comprise HO-1, thioredoxin,

glutathione reductase, glutathione peroxidase (GPx) and SOD1 (Thimmulappa *et al.*, 2002).

Heme oxygenase (HO) is an antioxidative and rate limiting enzyme involved in heme catabolism which consists of two active isoforms of HO: HO-1 and HO-2 (Ferrandiz & Devesa, 2008). HO-1 is expressed in an inducible manner whereas HO-2 is constitutively expressed (Kuwano *et al.*, 1994; Yet *et al.*, 2002). HO-1 functions to catalyse the degradation of heme generating metabolites such as carbon monoxide (CO), ferrous iron (Fe^{2+}) and biliverdin. Biliverdin is rapidly catalysed by bilirubin reductase to produce bilirubin (Otterbein *et al.*, 2003; Singleton & Laster, 1965). The byproducts produced from the breakdown such as CO and bilirubin act as a potent antioxidant. Due to its antioxidative effect, it exerts protective effects to prevent inflammation under stressed condition and cellular damage. Mechanistically, upon oxidative stress, activated Nrf2 transcribes HO-1 to prevent inflammation by attenuating iNOS with the aids of free iron and CO to block the release of NO (Wang *et al.*, 2004). Furthermore, free Fe2+ upregulates ferritin, an iron-chelating protein and promotes ATPase pump that eliminates intracellular Fe2+ from the cell. Ferritin averts the release of free radicals by binding free Fe2+ which involves in Fenton reaction to favor the production of ROS (Balla *et al.*, 1992; Ferris *et al.*, 1999; Otterbein *et al.*, 2003). Therefore, apart from HO-1, the byproducts also function as potent anti-inflammatory agents. It is well documented that NF-κB, a vital mediator of inflammatory response is closely associated with Nrf2/HO-1 signalling. Induction of Nrf2/HO-1 signalling degrades IKKβ through ubiquitination resulting in the inactivation of NF-κB and thus dampens inflammatory response (Nair *et al.*, 2008).

Therefore, activation of the phase II detoxification limits the inflammatory events from further exacerbating the oxidative stress-induced cell damage. However, the levels of

Nrf2 might be modulated during the progression of diseases despite the ROS accumulation. Several studies have proven that the activation of Nrf2 to translocate from cytoplasm to nuclear is absent in the brains of AD patients despite the presence of oxidative stress (Kanninen *et al.*, 2008; Ramsey *et al.*, 2007). Other than its role in endogenous defence, Nrf2 is considered an essential signalling mediator of inflammation in the brain. Therefore, it is targeted as a potential therapeutic candidate to combat neuroinflammation and neurodegenerative diseases (Buendia *et al.*, 2016).

2.2.4 Inflammatory mediators

2.2.4.1 Nitric oxide and inducible nitric oxide synthase

Nitric oxide (NO), a short lived free radical, is known to possess a wide range of biological functions (Aktan, 2004). This molecule freely diffuses within cells to transduce various physiological and pathophysiological events in various organ systems including CNS. The NO and L-citrulline are synthesized from L-arginine with N-hydroxy-L-arginine (NOH arginine) as intermediate catalysed by monooxygenase I and II (Figure 2.17). The synthesis of NO is catalysed by a family of enzymes, nitric oxide synthases (NOSs) (Alderton *et al.*, 2001; Knowles & Moncada, 1994; Yuste *et al.*, 2015). NOSs are mainly manifested by three isoforms encompassing of i) inducible NOS (iNOS) ii) neuronal NOS (nNOS) and iii) endothelial NOS (eNOS). nNOS and iNOS appear as soluble forms whereas eNOS is membrane bound, with its N-terminal myristoylated (Liu *et al.*, 1995). eNOS is expressed in motor neurons and astrocytes (Alderton *et al.*, 2001) while nNOS presents in astrocytes, synaptic spines and loose connective tissues in the brain (Saha & Pahan, 2006). Both nNOS and NOS are constitutively expressed in cells. In normal physiological conditions, iNOS is undetectable except under some circumstances during oxidative stress or inflammatory response when stimulated by tissue injuries or infections, whereby it is actively expressed in microglia and astrocytes

(Dawson & Dawson, 1998). Among NOSs, iNOS is primarily associated with inflammation and proposed to pathogenesis and progression of neuroinflammatory and neurodegenerative diseases (Saha & Pahan, 2006). The interactions between NO and superoxides can exert both oxidative effects by amplifying the formation of oxidants as well as antioxidative effects to limit oxidative damage via scavenging the superoxides (Lundberg *et al.*, 2015). On the contrary, oxidation, nitration and nitrosation can occur which lead to excessive accumulation of NO. Considerable evidence has demonstrated that the level of NO and iNOS are proximally corresponded to the degree of neuroinflammation. Aberrant production of NO and its derivatives trigger inflammatory response, loss of neurons, injuries and misfolding of proteins contributes in the development of AD (Saha & Pahan, 2006). In addition, increasing oxidative stress including nitrosative stress has been linked to the pathogenesis of neurodegenerative diseases as this reaction exacerbates the inflammatory response by enhancing the accumulation of Aβ in the development of AD (Nakamura & Lipton, 2011; Thiabaud *et al.*, 2013).

2.2.4.2 Cyclooxygenase-2 and prostaglandin E

Cyclooxygenase (COX) also known as prostaglandin G/H synthase, exists in two forms which are designated as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Morita, 2002). COX-1 is constitutively expressed in most tissues to regulate homeostasis including platelet aggregation, gastric cytoprotection and kidney function. Inducible COX-2 is not distributed in most normal tissues but it is expressed in the predominantly orchestrate the inflammatory responses (Hoffmann, 2000; Vane *et al.*, 1998). COX is a rate-limiting enzyme which involves a multi-step reactions. At the initial step, intermediate prostaglandin G_2 (PGG_2) is synthesized from arachidonic acid which is catalysed by the COX enzyme. PGG_2 is then transformed into prostaglandin H2

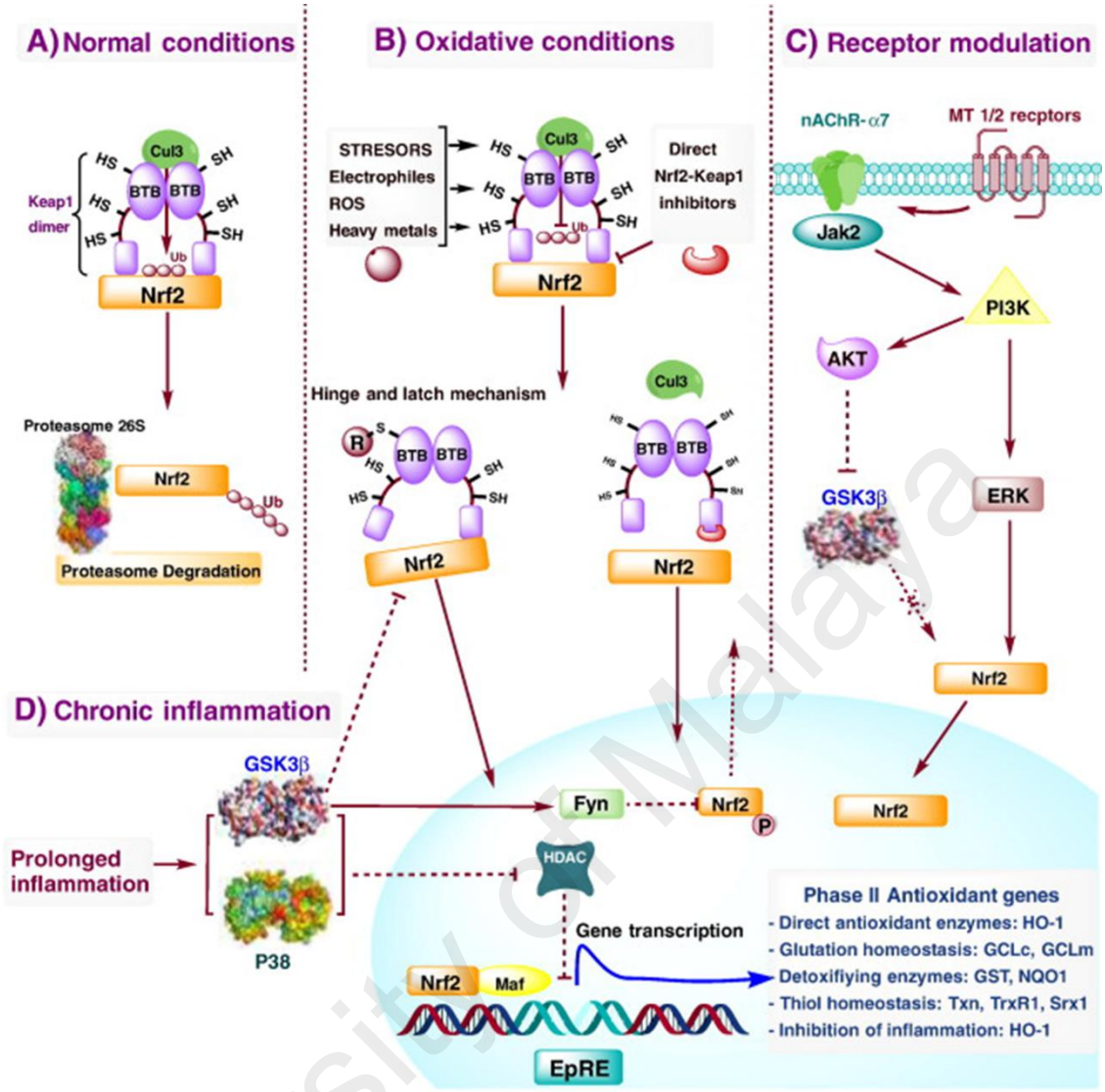


Figure 2.16: Nrf2 signalling pathway (Buendia *et al.*, 2016).

(PGH2) and the release of free radicals via a peroxidase reaction. Subsequently, PGH2 is converted into various prostaglandins such as PGE2, PGF2α, PGD2, prostacyclin and thromboxane B2 through tissue-specific synthases (Tuppo & Arias, 2005; Williams *et al.*, 1999). PGE2 also functions as a crucial homeostatic factor as well as an essential mediator of immunopathology in chronic infections, inflammation and cancer (Phipps *et al.*, 1991). The function of COX has been highlighted in regulating numerous inflammatory-related physiopathology including Alzheimer's disease, cancer, arthritis and cardiovascular diseases (Williams *et al.,* 1999). Excessive production of COX-2 and PGE2 proteins in the brain has been closely associated with the onset of inflammation which is a driving force of the AD pathogenesis via amyloidosis. Therefore, suppression of COX-2 becomes a therapeutic target in limiting the inflammatory response. This is supported by enormous epidemiological studies indicating that inhibition of COX-2 by NSAIDS and COXIBs (selective COX-2 inhibitors) may reduce the progression of Alzheimer's disease or delay its onset (Landi *et al.*, 2003).

2.12.3 Nuclear Factor Kappa B

Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is an ubiquitous regulator of a multitude of signalling pathways which is classified as one of the Rel family member of transcription factors. NF-κB belongs to the member of Rel family transcription factors which include five different members such as p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100 (Chen & Greene, 2004). Rel proteins consist of a Rel homology domain which has a conserved N-terminal region and DNA-binding/dimerization domain (Hayden & Ghosh, 2008). NF-κB proteins are able to form homodimers or heterodimers in transcribing the gene expression by binding to the DNA sequences of target genes within the enhancer and promoter regions (Perkins, 2007). NF-κB binding sites are known to transcribe a multitude of genes including adhesion,

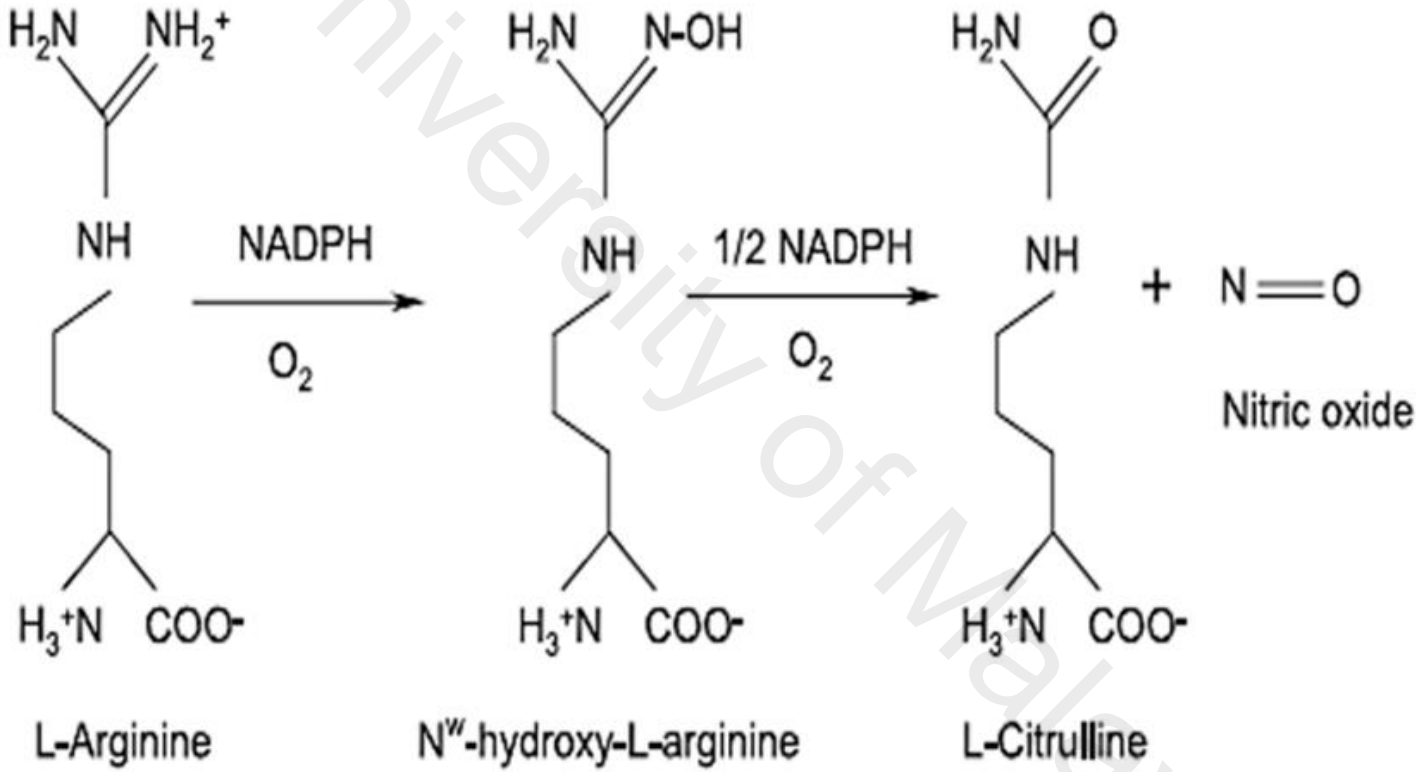


Figure 2.17: Production of nitric oxide from L-arginine. Adapted from Hollenberg and Cinel (2009).

molecules, cyclooxygenase-2, inducible NO synthase, cytokines (IL-1, IL-6 and TNF-α), angiogenic molecules and cell cycle regulatory molecules (Duh *et al.*, 1989; Gupta *et al.*, 2010) which orchestrated wide-ranging biological processes such as immune response, cell survival, cell proliferation, inflammation and cell cycle (Kaltschmidt *et al.*, 2005; Ledoux & Perkins, 2014; Sen & Baltimore, 1986). Under normal physiological conditions, NF-κB remains sequestered as latent form in the cytoplasm where an inhibitor protein, IκB is bound. The family members of IκB mainly consists of IκBα, IκBβ, IκBγIκBδ and IκBε (Whiteside & Israel, 1997). However, IκBα is the most common regulatory protein which exists in mammalian cells and binds to the p50/RelA heterodimer. The presence of ankyrin repeats in IκB binds to RHD in NF-κB proteins to mask the nuclear localization signal and hence prevents NF-κB translocation into nuclear compartment (Baeuerle, 1998). Activation of NF-κB is initiated by a plethora of stimuli, for instance, lipopolysaccharide (LPS), oxidative stress, inflammatory stimuli, tumour promoters, cytokines and UV light (Baeuerle & Henkel, 1994). The trigger by these stimuli leads to the phosphorylation of IκB by IκB kinase (IKK). IKK is distinguished by three distinct subunits such as IKK-α, IKK-β and IKK-γ in which IKK-α and IKK-β are catalytic subunits whereas IKK-γ is a regulatory subunit (NEMO) to detect and incorporate upstream activation signals (Lee & Hannink, 2002).

NF-κB can be classified into two main pathways which are classical or canonical pathway and alternative or non-canonical pathway (Figure 2.18). Canonical NF-κB pathway is the most extensively reported and is initiated via cell surface receptors (T-cell receptor (TCR), B-cell receptor (BCR), IL-1 receptor, Toll-like receptors and TNF receptor) in response to the pro-inflammatory cytokine exposure. Activated IKK complexes in turn activate the phosphorylation of IκB. Degradation of IκB is mediated via ubiquitinylation and proteolytic degradation by the proteasomes which facilitates the nuclear translocation of NF-κB. On the contrary, the non-canonical pathway is activated

by the TNF receptor superfamily, including CD40, the lymphotoxin β receptor, receptor activator of NF-κB and the BAFF receptor followed by accumulation of NF-κB inducing kinase (NIK). Activation of non-canonical pathway requires only the IKK-α subunit to culminate in the inactivation of IκB by phosphorylating and degrading p100 to yield p52. The protein p52 is then dimerized with RelB to translocate into nucleus (Hayden & Ghosh, 2004; Jost & Ruland, 2007). Thus, both pathways could lead to the degradation of IκB by respective IKK resulting in the activation NF-κB signalling pathway by releasing NF-κB into nucleus where it acts as a transcription factor by binding to the target genes (Chen *et al.*, 1998; Wan *et al.*, 2007).

2.2.4.3 Cytokines

Innate immune system is an utmost important first line defense to opsonize and remove apoptotic cells. Activation of innate immune systems triggers cells of the adaptive immune system to release various cytokines and chemokines that promotes the adhesion molecules on the blood brain barriers (BBB) and induces the activation of costimulatory molecules on microglia. Cytokines are released by various immune and non-immune cells including T-lymphocytes, natural killer cells, macrophages, schwann cells, glia cells, neuron cells and fibroblasts. Cytokines possess a host of functional roles in regulating cell growth, cell proliferation, inflammatory response and apoptosis (Rubio & Morillas, 2012).

Cytokines are pleiotropic molecules which can be classified into pro-inflammatory and anti-inflammatory cytokines. This molecule possesses prominent roles in regulating inflammatory responses to restore cell homeostasis in CNS (Nathan, 2002). Interferons, interleukins (ILs), chemokines, tumour necrosis factor (TNF), colony-stimulating factors (CSF) and growth factors are categorized as cytokines. Among the cytokines, interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor (TNF) are well studied as pro-

inflammatory cytokines whereas interleukin-4 (IL-4) and interleukin-10 (IL-10) are considered as anti-inflammatory cytokines (Kelso, 1998; Miller *et al.*, 2009). Interactions between pro-inflammatory cytokines and senile plaques act as a cardinal node in the progression of AD. Under normal physiological conditions, pro-inflammatory cytokines is generally maintained at low concentrations. On the contrary, aberrant secretion of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ is stimulated in response to oxidative stress, infection or injuries (Figure 2.19). The excessive production of pro-inflammatory cytokines may impair the neuronal function leading to cell death which exacerbates the progression of diseases such as AD (Lue *et al.*, 2001; Wang *et al.*, 2015).

IL-1α and IL-1β belong to the family of IL-1 cytokine which functions as an initiator to trigger the onset of inflammatory response leading to neuronal degeneration. IL-1α is mainly distributed in cytosol and IL-1β mainly appears as a soluble form (Dinarello, 1996; Netea *et al.*, 2010). IL-1β is synthesized by microglia and astrocytes and secreted as a pro-forms. Specifically, pro- IL-1β can be activated by inflammasomes into IL-1β via the cleavage of caspase-1 (Lamkanfi & Dixit, 2012). It is well documented that excessive secretion of IL-1β was observed in neurodegenerative conditions including AD. Production of IL-1β can be amplified by various stimuli such as lipopolysaccharide, prostaglandin E2 and Aβ (Allan *et al.*, 2005; Zipp & Aktas, 2006).

Interleukin-6 (IL-6), a glycoprotein, acts as a double edged sword which functions as pro-inflammatory and anti-inflammatory cytokine. IL-6 is involves in a myriad of biological responses including tissue regeneration (Cressman *et al.*, 1996; Grivennikov *et al.*, 2009), inflammation (Becker *et al.*, 2004; Rabe *et al.*, 2008) and pathogen defence (Hoge *et al.*, 2013). It plays an important role in central nervous system. IL-6 is predominantly produced by activated microglia and astrocytes in the human central nervous system. Additionally, aberrant secretion of IL-6 further stimulates microglia and

astrocytes following chronic activation of pro-inflammatory cytokines cascades (Querfurth & LaFerla, 2010). Empirical evidences has been revealed that this dysregulation of IL-6 in CNS results in neuroinflammation and neurodegeneration which is a proximal event in AD pathogenesis (Angelopoulos *et al.*, 2008; Campbell *et al.*, 1993; Fattori *et al.*, 1995). In contrast, IL-6 is suggested to act as anti-inflammatory and immunosuppressive molecule which is able to ameliorate the inflammatory conditions (Akaneya *et al.*, 1995; Tilg *et al.*, 1997). It has also shown to possess neuroprotective effect by improving the survival of neurons and protect neurons from excitotoxicity (Akaneya *et al.*, 1995; Hama *et al.*, 1991; Kushima & Hatanaka, 1992).

TNF-α is a membrane-bound protein which can be found in neuronal and glial cells. TNF-α functions as a key mediator in orchestrating the cytokine cascade upon the induction of inflammatory response. In addition, activation of TNF elicits a diverse cellular functions via apoptosis, NF-κB and MAPK signalling transduction pathways by binding to two cell surface receptors, TNFR1 and TNFR2 (Choi *et al.*, 2005; Montgomery *et al.*, 2013; Tartaglia & Goeddel, 1992). It has been reported that overexpression of pro-inflammatory cytokine, TNF-α which is present in the brain and plasma of AD patients has detrimental effect on neuronal viability and exacerbates the inflammatory response in concert with other pro-inflammatory signalling molecules (Breder *et al.*, 1993).

Production of pro-inflammatory cytokines can be counteracted by the release of anti-inflammatory cytokines which are important determinants for the balance between pro- and anti-inflammatory conditions. The most widely studied anti-inflammatory cytokines are interleukin-4 (IL-4) and interleukin-10 (IL-10) which are present in all glial cells. IL-10 is one of the most potent anti-inflammatory cytokines (Kim *et al.*, 2016). Both IL-4 and IL-10 can alleviate inflammation by repressing the synthesis of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β and thereby inhibiting receptor activation in the

brain (Brown & Hural, 1997; Marchant *et al.*, 1994; Wang *et al.*, 1995). Exposure to high concentrations of anti-inflammatory cytokines results in a negative-feedback loop that limits the glial activation and the progression of AD (Kiefer *et al.*, 1993; Zhang & An, 2007).

2.3 *Elephantopus scaber*

Since the dawn of human civilization, human beings have relied and made use of different parts of the plants by different cultures for treating diverse of ailments and myriad of diseases. To date, natural products such as fruits, vegetables and medicinal plants which have been the main source in traditional medicine remain as an important source of new drug leads in curing a wide spectrum diseases such as cardiovascular diseases, diabetes mellitus and cancer (Cragg & Newman, 2013; Unnati, 2013). *Elephantopus scaber* L., also known as Prickly-leaved elephant's foot or di dan tou in Chinese or tapak sulaiman in Malay, belongs to the family of Asteraceae. The genus *Elephantopus* contains about 32 species among these including *Elephantopus mollis, Elephantopus scaber, Elephantopus tomentosus* and *Elephantopus carolinianus*. It is a scabrous wild weed which forms a spreading rosette in shady places. It is a coarse and hairy herb with about four purple flowers in a cluster. It grows with a height of 30-60cm. This herb plant can be widely found in various continental including Europe, Asia (India, Malaysia, Indonesia, Japan, Thailand, Hong Kong and Taiwan), America and Africa (Ho *et al.*, 2009).

In the past, *E. scaber* is a medicinal herb where a whole plant from leaves to roots has been traditionally applied to treat a variety of diseases in different countries. Each country has different traditional practices against diverse diseases. For instance, in Brazil, it is used as a tonic to treat cough, bronchitis and asthma. In Taiwan and China, the whole plant is applied to treat hepatitis, bronchitis, pneumonia and fever. In Malaysia, the roots

of *E. scaber* has been prepared as decoction to prevent inflammation after childbirth and induce the abdominal part contraction (Ho *et al.*, 2009). According to the report, Murut people, the native of Sabah, Malaysia often used the roots of *Elephantopus* species to cure bloody stool (Kulip, 2003). In India, leaves or whole plant was used to treat menorrhagia, tetanus and wounds whereas the root was used for the treatment of spermatorrhea and menorrhagia (Behera & Misra, 2005; Kumar *et al.*, 2007). On the other hand, it is also deployed as an Ayurveda medicine to treat minor neoplasm or cancer (Balachandran & Govindarajan, 2005).

Over the last two decades, the wide ethnomedicinal values of *E. scaber* has raised great attention among the scientists and led to extensive scientific investigation in various model of diseases. It is well documented that *E. scaber* exhibited various pharmacological activities including anticancer (Farha *et al.*, 2015; Geetha *et al.*, 2010; Kabeer *et al.*, 2013; Pitchai *et al.*, 2014; Su *et al.*, 2011; Xu *et al.*, 2006), anti-inflammatory (Abhimannue *et al.*, 2016; Hung *et al.*, 2011), wound healing (Aslam *et al.*, 2016; Singh *et al.*, 2005), antidiabetic (Daisy *et al.*, 2009), antimicrobial, anti- bacterial (Anitha *et al.*, 2012; Kumar *et al.*, 2004), hepatoprotective (Ho *et al.*, 2012; Rajesh & Latha, 2001), antiasthmatic (Sagar & Sahoo, 2012) and antioxidant (Aslam *et al.*, 2016). *E. scaber*, an ayuverdic medicine, is one of the alternative medicine which utilized against cancer and leukemia in India (Balachandran & Govindarajan, 2005). This was further supported by a number of scientific reports which revealing *E. scaber* exhibited potent cytotoxic effect against different cancer cell lines such as mammary adenocarcinoma, breast cancer, lung cancer, leukemia, nasopharyngeal cancer and cervical carcinoma cells (Huang *et al*., 2010; Ichikawa *et al*., 2006; Kabeer *et al*., 2013; Su *et al*., 2011).

Secondary metabolites from natural products including plants have been the source of lead molecules in drug development due to their growing diversity and high level of

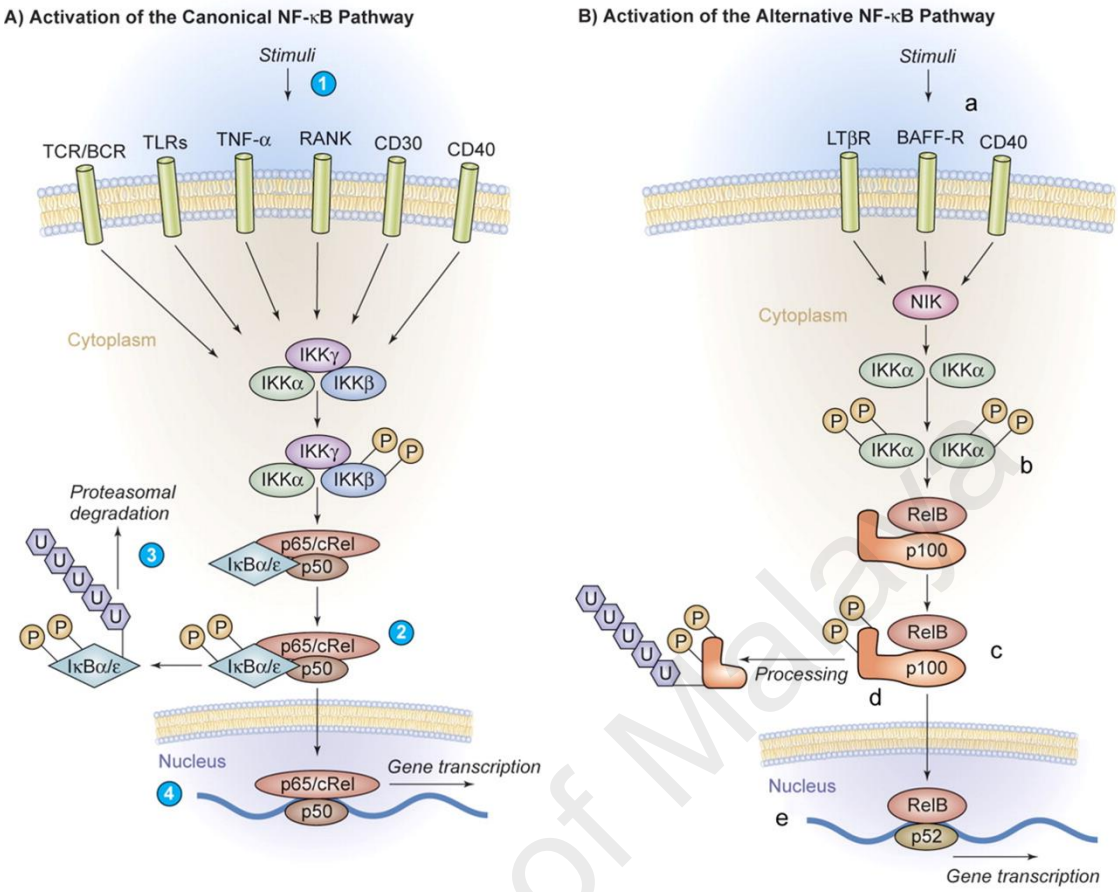


Figure 2.18: Canonical and non-canonical alternative NF-κB pathway**s**. Adapted from Jost and Ruland (2007).

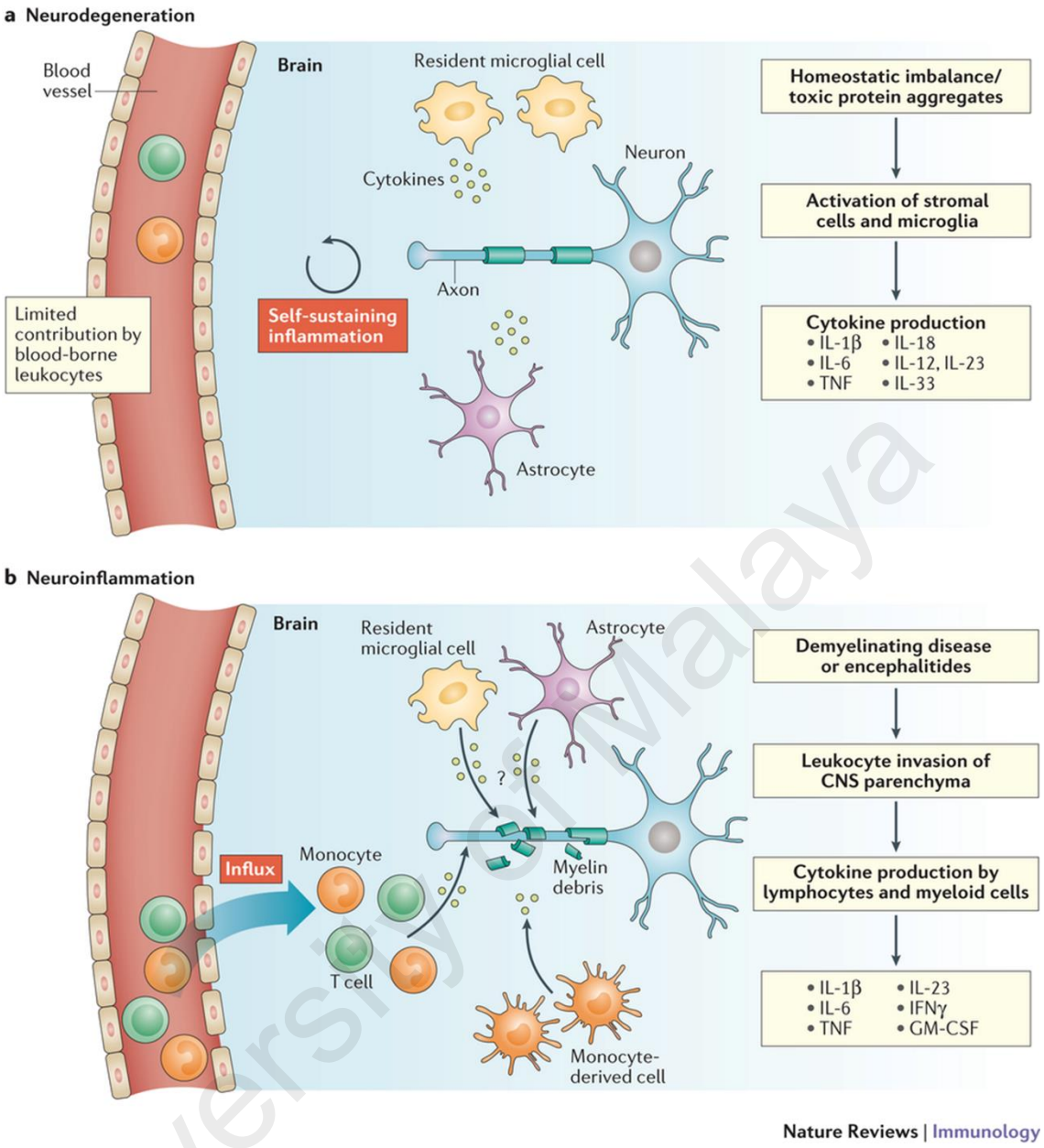


Figure 2.19: The network of cytokines in the brain during neurodegeneration and neuroinflammation (Becher *et al.*, 2017).

specificity in biological activities, either additively or synergistically. Moreover, secondary metabolites generally can be categorized into several main classes including phytosterols, alkaloids, terpenoids, polyphenols and organosulfur compounds. Numerous studies have identified different classes of phytochemicals in *E. scaber* consisting of terpenoids, flavonoids, minerals, salt, phenolic acids, essential oils and steroids. The compounds belonging to terpenoids, flavonoids, phenolic acids, steroids and sesquiterpene lactones were summarized in Table 2.1 and Figure 2.20. Besides, a number of essential oils were identified in *E. scaber* such as cyclosativene, copaene, isopropyl dimethyl hexahydronaphthalene,trimethyl imethylenedecahydronaphthalene, zingiberene, β-caryophyllene, caryophyllene, dimethyl-6-(4-methyl-3-pentenyl)-2-norpinene, β-sesquiphellandrene, isocaryophyllene, α-santalol, ledol, α-bisabolol, caryophyllene oxide, β-bisabolol, isopropyl dimethyl tetrahydronaphthalenol, cadinol, hexahydrofarnesyl acetone, hexadecanoic acid, phytol and octadecadienoic acid (Wang *et al.*, 2004). In addition, presence of potassium chloride salt and minerals such as calcium, magnesium, iron and zinc was also detected in *E. scaber*. Another study reported that a trace of elements was identified including Si, Ca, Cl, Mg, S, K and P in the leaves of *E. scaber* while Al, Fe, Ti, Sr and V in roots (Santhosh, 2012).

Sesquiterpene lactones (SLs) is sub-classified under terpenoids which are predominantly derived from plants of Asteraceae family. Notably, sesquiterpene lactones with a wide spectrum of biological activities including anticancer, anti-inflammation, anti-bacterial and antifungal has been verified by considerable scientific evidences (Canales *et al.*, 2005; Chadwick *et al.*, 2013; Gach *et al.*, 2015). Deoxyelephantopin (DET), a sesquiterpene lactone from *E. scaber* has been found to corroborate previous reports evidenced by its multifaceted biological activities. Specifically, mounting scientific evidence demonstrated that DET significantly confers anticancer effect against various cancer cell lines such as mammary, cervical, prostate, lung, liver and

nasopharyngeal (Farha *et al.*, 2014; Huang *et al*., 2010; Kabeer *et al*., 2013; Mehmood *et al*., 2016; Su *et al.*, 2011). It has been known to be a multi-functional mediator which mediates cell death via multiple signalling transductions such as cell cycle, apoptosis, PI3K/Akt, nuclear factor kappa B, signalling transducer and activator of transcription 3 (STAT) as well MAPK signalling pathways (Farha *et al.*, 2014; Huang *et al.*, 2010; Kumar *et al.*, 2014; Mehmood *et al.*, 2017). Hence, current trend has geared towards multi-targeted drugs derived from natural products which can mediate multiple signalling pathways to target various diseases. Thus, this emerging phenomenon has prompted interest in the discovery of multi-targeted natural-derived phytochemicals as potential therapeutic agents for intervention as well as to circumvent the drawback of single-targeted drug (Khan & Mukhtar, 2008; Talevi, 2015; Teiten *et al.*, 2010; Zhu *et al.*, 2015).

To the best of our knowledge, the effect of *E. scaber* on human colorectal carcinoma cells and LPS-induced microglia cells so far has not been investigated and its mechanisms remain unknown. The aim of the present study is to elucidate anticancer and anti-neuroinflammatory effect of *E. scaber* in human colorectal cancer cells and LPS-induced microglia cells, respectively.

The specific objectives of this study were:

1. To determine the *in vitro* cytotoxic activity of ethanol extract, hexane, ethyl acetate and aqueous fractions of *E. scaber* and investigate the underlying apoptotic mechanisms against human colon cancer cells.
2. To isolate and identify the bioactive compound(s) from ethyl acetate fraction of *E. scaber* via bioassay-guided approach which is responsible for the growth inhibitory effects.

3. To investigate the *in vitro* and *in vivo* anti-tumour effects and elucidate the underlying cell death mechanisms conferred by deoxyelephantopin in HCT116 cells human colon carcinoma.
4. To evaluate the anti-neuroinflammatory properties of ethyl acetate fraction of *E. scaber* in LPS-induced BV-2 microglia cells.

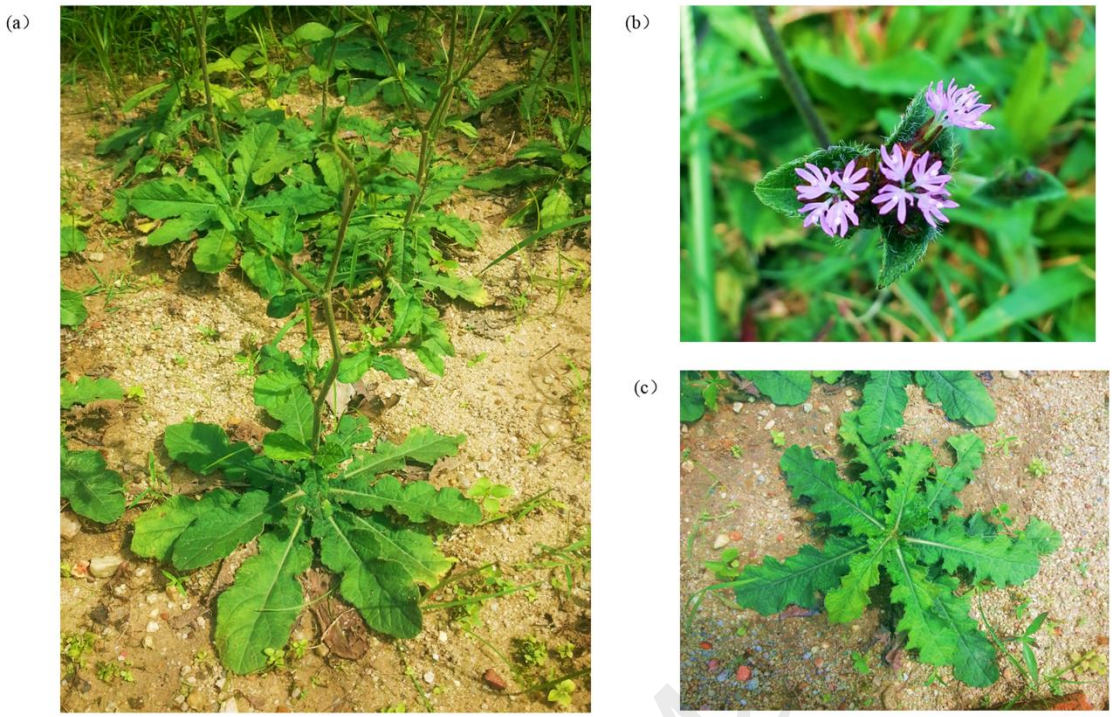


Figure 2.20: *Elephantopus scaber* (A) whole plants, (B) flowers and (C) leaves.

Table 2.1: Phytochemicals isolated from *E. scaber*

Class	Plant part	Examples	References
	Whole plant	p-coumaric acid	(Chang, 2011)
	Whole plant	vanillic acid	(Huang, 2009)
	Whole plant	syringic acid	(Huang, 2009)
	Whole plant	isovanillic acid	(Huang, 2009)
	Whole plant	p-hydroxybenzoic acid	(Zhang, 2011)
	Whole plant	ferulic acid	(Huang, 2009)
	Whole plant	3-methoxy-4-hydroxyl cinnamaldehyde	(Huang, 2009)
	Roots	Tricin	(Su *et al.*, 2009)
	Aerial part	Trans-p-coumaric acid	(Chang, 2011)
	Aerial part	Methyl trans-caffeate	(Chang, 2011)
	Aerial part	indole-3-carbaldehyde	(Chang, 2011)
	Aerial part	Trans-caffeic acid	(Chang, 2011)
	Aerial part	methyl 3,4-dicaffeoylquinate	(Chang, 2011)
Flavonoids	Aerial part	luteolin-7-O glucuronide 6-6”-methyl ester	(Chang, 2011)
	Aerial part	Luteolin-4-o-β-d glucoside	(Chang, 2011)
	Aerial part	Luteolin	(Chang, 2011)
Sesquiterpenoids	Whole plant, roots	Deoxyelephantopin	(But, 1997; Than, 2005)
	Whole plant, roots	isodeoxyelephantopin	(But, 1997; Than, 2005)
	Whole plant	Scabertopin	(But, 1997)
	Whole plant	isoscabertopin	(Liang *et al.*, 2008)
	Whole plant	17,19 dihyrodeoxyelephantopin	(Than, 2005)
	Whole plant	iso-17,19 dihyrodeoxyelephantopin	(Than, 2005)
	Whole plant	11,13 dihyrodeoxyelephantopin	(de Silva, 1982)
	Whole plant	elescaberin	(Liang *et al.*, 2008)
	Whole plant	deacylcyanopicrin	(Hisham *et al.*, 1992)
	Aerial part	Scabertopinol	(Chang, 2011)

Table 2.1, Continued

	Root	Glucozaluzanin-c	(Hisham *et al.*, 1992)
	Root	Deacylcyanopicrin 3	(Hisham *et al.*, 1992)
	Root	β-glucopyranoside crepiside E	(Hisham *et al.*, 1992)
Triterpenoids	roots	Lupeol	(Sim, 1969; Su *et al.*, 2009)
	Whole plant	Betulinic acid	(Liang, 2007; Su *et al.*, 2009)
	Whole plant	30-hydroxylupeol	(Liang, 2007)
	Whole plant	Lupeol acetate	(Liang, 2007; Su *et al.*, 2009)
	Whole plant	Ursolic acid	(Liang, 2007; Su *et al.*, 2009)
	Whole plant	Friedelin	(Liang, 2007)
	Whole plant	Epifriedelinol	(Liang, 2007; Sim, 1969)
Sterols	Whole plant	Ursa-12-ene-3β-heptadecanoate	(Liang, 2007)
	Whole plant	Stigmasterol	(Sim, 1969)
	Whole plant	Stigmasterol-3-O- β-D-glucoside	(Hisham *et al.*, 1992)
	Whole plant	β-sitosterol	(Zhang, 2011)
	Whole plant	Daucosterol	(Zhang, 2011)
	whole plant	28Nor-22(R) witha 2,6,23-trienolide	(Daisy *et al.*, 2009)

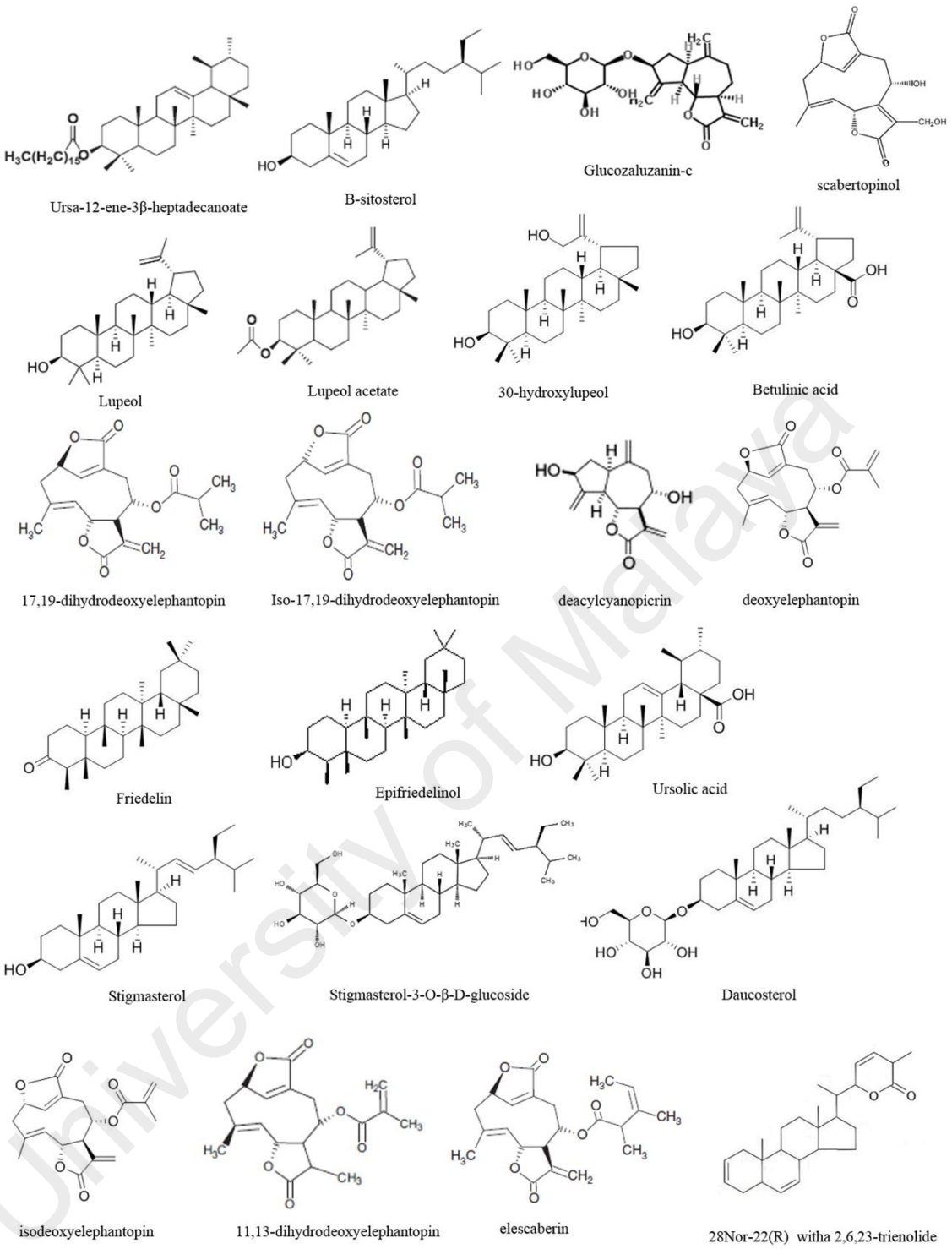


Figure 2.21: Structures of phytochemicals of *Elephantopus scaber*.

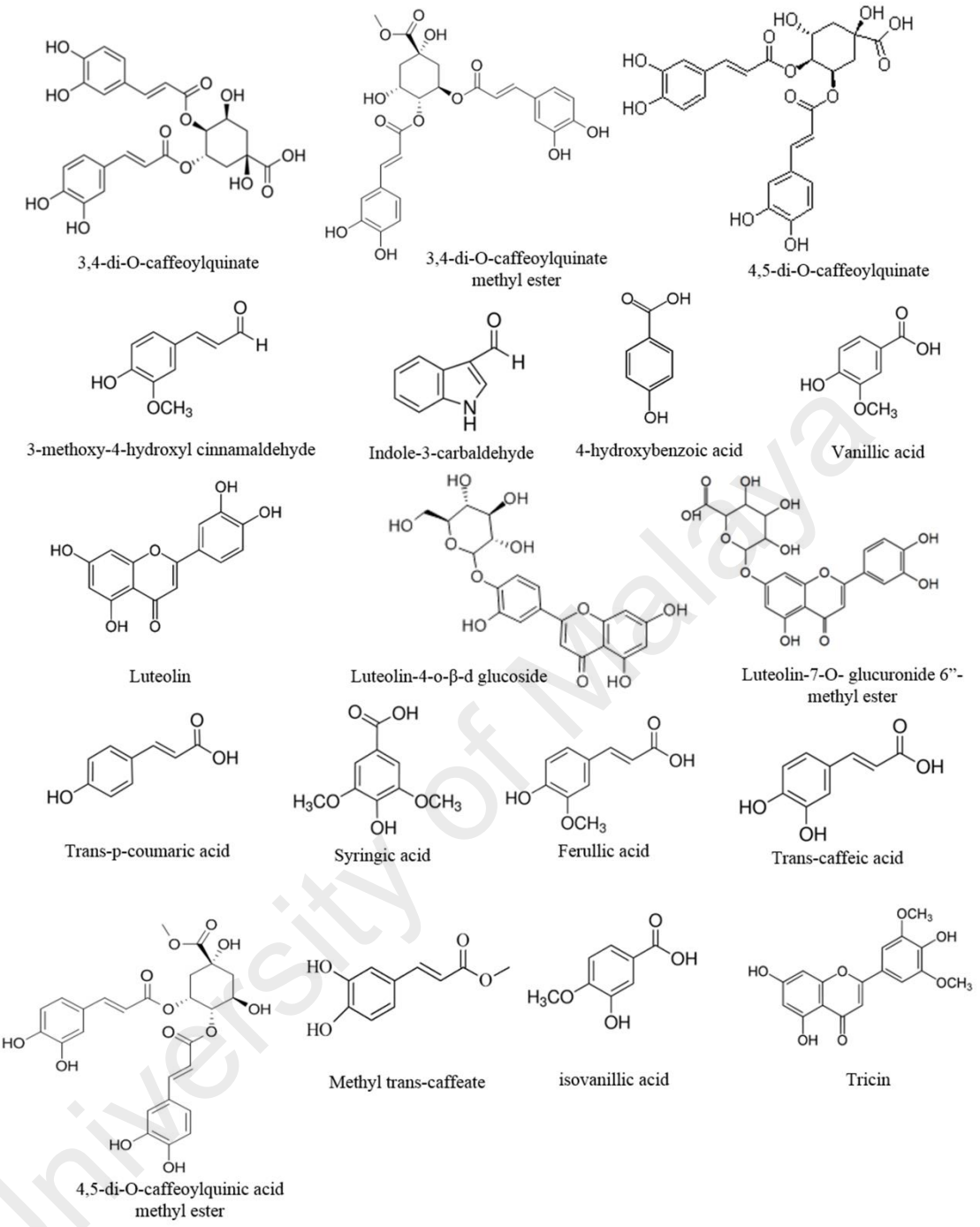


Figure 2.21, Continued.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

HT-29, HCT116 human colorectal cancer cancer cells and CCD841-CoN cells human normal colon cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM) and Medium and Eagle's Minimum Essential Medium (EMEM) were purchased from Sigma, St Louis, MO, USA. Foetal bovine serum (FBS), penicillin streptomycin and amphotericin B were from PAA Lab, Pasching, Austria. Accutase was purchased from Innovative Cell Technologies (San Diego, CA, USA). Antibiotic-antimycotic and TrypLE were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Sodium bicarbonate (Na_2CO_3), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO**),** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Phosphate buffer (PBS), Hoechst 33342, Propidium iodide, 2'7'-dichlorofluorescein diacetate (DCFH-DA), tert-butyl hydroperoxide (TBHP), tryphan blue, propidium iodide (PI), acridine orange (AO), 10% buffered formalin solution, hematoxylin, lipopolysaccharide, SOD determination kit, catalase kit, 10% buffered formalin solution, Eosin Y, protease and phosphatase inhibitor cocktails were purchased from Sigma (St louis, MO, USA). 95% Ethanol, absolute ethanol, ethyl acetate, hexane, preparative thin layer chromatography, silica gel (60-200 mesh), tween-20 detergent and skim milk were purchased from Merck. were purchased from Merck. Annexin V-fluorescein-isothiocyanate (FITC), 10 x Annexin V binding buffer amd Cycletest™ Plus DNA Kit were purchased from BD-Bioscience (Erembodegem, Belgium). FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody, anti-human Bax polyclonal antibody, IgG1 isotype control, Cytofix/Cytoperm kit was purchased from BD Biosciences (San Diego, CA, USA). FITC-

conjugated goat anti-rabbit F(ab')2 polyclonal secondary antibody were purchased from Abcam (Cambridge, UK). Primary antibodies such as cleaved PARP, cleaved caspase-3, p-p53, p21, CDK2, CDK4, CDK 6, cyclin A2, cyclin D1, cyclin E, Akt, p-Akt (Ser473), p-Akt (Thr308), p PTEN, p PDK-1, mTOR, p mTOR, cleaved caspase-3, procaspase-8, -9 , -10, cleaved PARP, XIAP, survivin, livin, Bax, Bak, Bcl-2, Bcl-xL, DR5, Fas, p-p38, p38, p-JNK, JNK, p-ERK1/2, ERK1/2, LC3A/B, Atg5, Atg7, p-p38 MAPK, p38 MAPK and COX-2, GAPDH and anti-mouse/rabbit immunoglobulin G-horseradish peroxidase-conjugated secondary antibody were purchased from Cell Signalling Technology (Danvers, MA, USA). Anti-mycotic solution, pierce western blotting filter paper, pierce nitrocellulose membrane (0.45 µm), RIPA buffer NE-PER^R nuclear and cytoplasmic extraction reagents, total nitric oxide (NO) assay kit and Prostaglandin E2 (PGE_2) Competitive ELISA kit, primary monoclonal antibodies iNOS, NF-κB p65, Nrf2, HO-1, lamin B and β-actin were purchased from Thermo-Scientific (MA, USA). Quick Start Bradford 1x Dye Reagent, 10x Tris/Glycine/SDS, Tris, Glycine, Sodium Dodecyl Sulfate (SDS), Resolving Gel buffer, Stacking Gel Buffer, Ammonium Persulfate, TEMED, 30% Acrylamide/Bis Solution, Precision plus protein Western C standards, 0.45µM nitrocellulose membrane, 0.2µM nitrocellulose membrane, filter paper and Clarity Western ECL kit were purchased from Bio-Rad. Mito ID membrane potential detection kit was purchased from Enzo life science (Lausen, Switzerland). Bovine serum albumin (BSA) was purchased from Amresco. RayBio ® Human Apoptosis Antibody Array kit purchased from RayBiotech (Norcross, GA, USA).

3.2 Methods

3.2.1 Preparation of the extract and fractions of *E. scaber*

The leaves of *E. scaber* were provided by a local supplier and authenticated by Dr. Yong Kien Thai. The voucher specimen (No.KLU47976) was deposited at the herbarium in the Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia.

First, the dried leaves of *E. scaber* (1.5 kg) were grounded and extracted with 70% ethanol at room temperature for three days to obtain ethanol crude extract (ESEE). The extract was then filtered by using the WhatmanNo.1 filter paper. The extraction were repeated again twice. All the collected filtrate was evaporated by using a rotary evaporator (Buchi) under reduced pressure at 40 ºC. 50g of dark green crude ethanolic extract was obtained. This was further proceed to partitioning step by using hexane to obtain *E. scaber* leaves' hexane fraction (ESHF). Subsequently, the hexane insoluble residue was further partitioned to yield an ethyl acetate-soluble fraction and aqueous fraction by using ethyl acetate and water (1:1) at room temperature. The ethyl acetate-soluble filtrate was concentrated to yield *E. scaber* leaves' ethyl acetate fraction (ESEAF) under pressure at 45 ºC whereas the aqueous filtrate was lyophilized to yield *E. scaber* leaves' aqueous fraction (ESAF). All the crude and fractions was dissolved in DMSO and filtered using 0.22 µm filter membrane. Throughout all the experiments, the final concentration of DMSO is remained below 0.5 % v/v.

3.2.2 Cell culture

HT29, HCT116 human colorectal cancer cells and CCD841-CoN human normal colon cells were grown in RPMI 1640 and EMEM medium which supplemented with 10% FBS, 100 mg/mL penicillin streptomycin and 50 mg/mL amphotericin B. The medium was filtered by using a sterilized 0.22 µm filter membrane (Minisart, Sartorius Stedim, Goettingen, Germany). The cells were sub-cultured every two days and detached from culture flasks by using Accutase. The cells were collected and centrifuged at 1000 rpm for 5min. The cell pellet was resuspended with medium, added into flask and maintained in an environment of 5% CO_2 at 37 ºC.

3.2.3 Cryopreservation and cell revival

Upon the cells reached 80 to 90% confluency, cells were harvested and centrifuged at 1000 rpm for 5 min. The pellet which dissolved with freezing medium containing 70% of medium, 20% of FBS and 10% of DMSO was then aliquoted to the sterile cryovials and labeled accordingly to the type of cell line, date and passage number. Subsequently, the vials were first kept in freezer (-20 °C) and then kept in liquid nitrogen storage vessel for longer storage. For cell revival, cryovials were thawed immediately in 37 °C water bath. Cells were aliquoted to conical centrifuge tubes containing medium and proceeded to centrifugation. Medium was added to cell pellet and transferred into 25cm^2 cell culture flask. The cells were left adhered in CO_2 incubator and removed the medium after 24 h to eliminate traces of DMSO prior to the replacement of fresh medium. The cells were used for experiments after at least 3 passages.

3.2.4 *In vitro* MTT cytotoxic assay

The *in vitro* cytotoxic effect of *E. scaber* against normal colon and colorectal cancer cells was measured by utilizing MTT assay. Viable cells in a density of 5 x 10^3 were seeded into 96-well plates and left attachment overnight. The cells were exposed to increasing concentrations of ESEE, ESHF, ESEAF, ESAF and 5-fluorouracil (5-FU) as positive control (0.20 - 200.00 µg/mL) for 72 h. Cells were exposed to DMSO without sample as negative control. Next, each well was added 20 µL of MTT (5mg/mL) and incubated for 4 h at 37 ºC. The medium was discarded and 150 µL of DMSO was added to resuspend the formazan crystals. The absorbance of the formazan product was quantified at a wavelength of 570nm and 650nm as a background by using microplate reader (Asys UVM340, Eugendorf, Austria). The percentage of cell viability was calculated base the following formula: percentage of cell viability (%) = (Absorbance of treated cells/absorbance of untreated cells) x 100%.

3.2.5 Detection of Nuclear Morphology via Hoechst 33342/PI staining

Based on the MTT results, ESEAF was subjected for further evaluation on the apoptosis inducing effects. Alteration of nuclear morphology can be distinguished by Hoechst 33342 and PI. Hoechst 33342 is a fluorescence dye that cell-permeant and can be detected by fluorescence microscope. Hoechst 33342 stains the viable cells with lower blue fluorescence and the apoptotic cells are brightly stained. Propidium iodide is membrane non-permeable dye to live cells which intercalating between the DNA breaks. 1x 10^6 HCT116 cells were seeded in 60 mm^2 culture dishes for 24 h followed by treatment with or without 10 µg/mL of ESEAF at different incubation period (6, 12 and 24 h). After incubation, the cells were harvested and rinsed with PBS. Addition of Hoechst 33342 (40 µg/mL) and Propidium Iodide solution (10 µg/mL) to stain cells for 30 min in the dark following by detection using inverted fluorescence microscope (Leica DM1600B, Wetzlar, Germany).

3.2.6 Phosphatidylserine Externalization by Annexin V and PI Staining

Translocation of phosphatidylserine to outer leaflet membrane is one of the earliest apoptotic event. This event can be detected by using Annexin V and PI staining which performed based on the manufacturer's instruction. The viable (Annexin V and PI negative stained), early apoptotic (Annexin V positive and PI negative stained), late apoptotic (both Annexin V and PI positive stained) and necrotic cells (Annexin V negative and PI positive stained) can be detected by using BD Accuri flow cytometry. 1 × 10^6 cells of HCT116 cells were seeded in 60 mm^2 culture dishes for overnight and were exposed to ESEAF (2.5, 5.0 and 10.0 µg/mL) while negative control was exposed to DMSO for 24 h. After treatment, both adherent and suspended cells were collected and centrifuged at 1800 rpm for 5 min harvested. The cell pellet was washed twice and resuspended in 1mL of 1× Annexin V binding buffer. 100 µL of cell suspension were

aliquoted in 5ml falcon round bottom tubes. The cells were stained with Annexin V-fluorescein-isothiocyanate (FITC) and PI (50 µg/mL) for 30 min in the dark followed by the addition of 1× Annexin V binding buffer (400 µL). The fluorescence intensity of the cells was measured in FL1-A (x-axis) and FL2-A channel (y-axis).

3.2.7 Detection of DNA fragmentation

A Terminal Deoxynucleotidyl Transferase UTP Nick End Labelling (TUNEL) assay which can detect DNA fragmentation was performed according to the manufacturer's protocol (Sigma). HCT116 cells were seeded and exposed to ESEAF or vehicle DMSO. After treatment, cells were collected, washed and fixed with 1% (w/v) paraformaldehyde in PBS for 15 min on ice. The cells were washed and followed by the addition of DNA labelling solution and incubation for 60 min at 37 °C. Later, the cells were stained with FITC-conjugated anti-BrdU antibody for 30 min in the dark followed by the addition of propidium iodide/RNase A solution for another 30 min in the dark. The fluorescence intensity emitted by cells was quantified by using Accuri C6 flow cytometer.

3.2.8 Determination of Mitochondrial Membrane Potential

JC-1, a fluorescent cationic dye is utilized for the assessment of mitochondrial membrane potential. HCT116 cells were cultured in 60 mm^2 culture dishes for 24 h prior to the treatment with ESEAF (2.5, 5.0 and 10.0 µg/mL). After 24 h, the cells were collected and rinsed with PBS. Medium containing JC-1 was added and stained the cells for 15 min at 37 °C. After staining, cells were centrifuged, washed and re-suspended with medium. The intensity of green and red fluorescence at FL1-A and FL2-A channels were measured by flow cytometry analysis.

3.2.9 Evaluation of Bax and Bcl-2 protein expression

Immunofluorescence staining was used to determine the protein expression of Bcl-2 family member including Bax and Bcl-2 via using flow cytometer. 1×10^6 cells which were treated with ESEAF or DMSO were collected and washed twice with PBS. Fixation and permeabilisation was done by using Cytofix/cytoperm kit. Addition of fixation/permeabilisation solution to the cells for 20 min incubation at 4 °C. Perm/Wash buffer was used for washing and cells were incubated for 15 min in this buffer. For each proteins, perm/wash buffer containing the respective antibodies was added to the cells. For direct Bcl-2 protein staining, the cells were stained with FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody or IgG1 isotype control for 30 min at 4 °C. For indirect Bax staining, the cells were stained with either rabbit anti-human Bax polyclonal antibody or IgG1 isotype control for 30 min at 4 °C followed by the FITC-conjugated goat anti-rabbit F(ab')2 polyclonal secondary antibody staining for 30 min at 4 °C. The cells were subjected to washing step by using Perm/Wash buffer. The fluorescence intensity was quantified at FL1-H channel by using flow cytometer.

3.2.10 Evaluation of caspase-3/7 and -9 activities

To detect caspase-3/7 and -9 activity, cells were exposed to ESEAF at different incubation periods and subjected to fluorescence staining. After treatment, collected cells were subjected to staining with 30× FLICA solution (caspase 3/7-FAM-DEVD-FMK; caspase 9-FAM-LEHD-FMK) for 1 h at 37 °C in dark followed by washing steps with wash buffer and re-suspending cell pellet with wash buffer. The fluorescence intensity of caspase-3/7 and caspase-9 which detected at FL1-A channel was analysed by flow cytometer.

3.2.11 Assessment of the Intracellular Reactive Oxygen Species (ROS) level

The fluorescent dye 2'-7'-dichlorofluorescein diacetate (DCFH-DA) detects the

accumulation of ROS. 1×10^6 cells were exposed to ESEAF (1.25 μg/mL to 10 μg/mL) or vehicle DMSO (negative control) or *tert*-butyl hydroperoxide (TBHP) as positive control for 4 h. After treatment, the cells were rinsed and stained with 50 μM DCFH-DA for 1 h. Once staining completed, collected cells were rinsed and resuspended with PBS. The DCFH fluorescence intensity was quantified by flow cytometer at FL1-A channel.

3.2.12 Investigation the protein expression of p53 Tumour Suppressor

The p53 protein expression was measured by immunofluorescence staining using flow cytometer. Cells were treated with or without 10 μg/mL of ESEAF at 6, 12 and 24 h. Cells were then collected and rinsed with PBS twice followed by fixation and permeabilisation using Cytofix/Cytoperm. Kit at 4 °C for 20 min. Then, the cells were subjected to washing steps twice with Perm/Wash buffer and Perm/Wash buffer was added for another 15 min incubation. The cells were subjected to the staining which incubated with FITC-conjugated mouse anti-human p53 monoclonal antibody or IgG2b isotype control for 30 min at 4 °C in the dark. Prior to the analysis by using BD AccuriC6 flow cytometer, the cells were rinsed with Perm/Wash buffer.

3.2.13 Western blot analysis

1×10^6 cells were seeded in 60 mm^2 culture dishes and underwent treatment with 2.5 to 10.0 μg/mL of ESEAF for 24 h. After treatment, both adherent and suspended cells were harvested, washed with cold PBS followed by centrifugation at 1800 rpm for 5 min. Subsequently, cold RIPA buffer consisting of protease and phosphatase inhibitors was added to cell pellets and incubated on ice for 5 min. The cells were then centrifuged at 14,000 × g for 15 min at 4°C. After centrifugation, the supernatant was kept in freezer. Bradford assay was used to assess the protein content of supernatant. 25 μg of protein were electrophoresed on the 12 % SDS-PAGE gel followed by the transfer of protein onto

a nitrocellulose membrane and blocking by using skim milk for 1 h. Membranes were then probed with primary antibodies (cleaved PARP and GAPDH) at 4°C overnight. After incubation, the membrane was proceeded to washing with TBST (0.05% Tween 20 in TBS) for thrice and probed with anti-mouse/rabbit secondary antibody for 1 h. Next, washing steps were repeated as previous prior to the staining by using enhanced chemiluminescence (ECL) detection kit. The band intensity was captured by gel documentation system followed by quantitative analysis with Vilber Lourmart.

3.2.14 Phytochemical profiling by GC–MS analysis

Gas chromatography-mass spectrum (GC-MS) analysis was carried out by using Agilent Technologies 7890A equipped with HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0 m x 250 μm x 0.25 μm, 7000 Mass Selective Detector and helium was utilized as carrier gas at 1 mL/min. In the beginning, the temperature of column was set at 100 ºC for 10 min, followed by increasing 5 ºC per minute to 300 ºC and kept isothermally for 45 min. The MS was operating at 70 eV. The phytochemicals were identified based on their mass spectral data from NIST 08 Spectral Library. The mass spectral fragmentation pattern which was match 90% and above was accepted. The known phytochemicals, deoxyelephantophin and isodeoxyelephantopin were used reference for validation.

3.2.15 Bioassay-guided approach to isolate active phytochemicals from the ethyl acetate fraction of *E. scaber*

Based on the previous results obtained, 11 g of ESEAF was fractionated via column chromatography (CC) made up of silica gel (60-200 mesh) utilizing dry packing method. The column was eluted via the following gradient solvent system of hexane: ethyl acetate (7:3 to 0:10) and ethyl acetate: methanol (8:2 to 0:10) to obtain nine fractions (F1-F9)

(Figure 4.1). Each collected fraction was subjected to evaluate cytotoxicity via MTT assay. Fraction F2 revealed as the most potent fraction with IC_{50} values of 0.95 ± 0.12 µg/mL. Thus, fraction F2 was further proceeded for purification via preparative thin layer chromatography (PTLC) followed by three times of elution with hexane: ethyl acetate (3:7), which led to compound **1** (0.18 g, R_f = 0.75) and compound **2** (0.29 g, R_f =0.70). The cytotoxicity of these two compounds were proceeded via MTT assay.

3.2.16 *In vitro* cytotoxicity via MTT assay

The *in vitro* cytotoxic effect of each fraction and compounds against normal colon and colorectal cancer cells was measured by utilizing MTT assay. HCT116 cells were plated and incubated in varying concentrations of each fraction or compounds (0.38-25 µg/mL) which resuspended in DMSO while 5-fluorouracil (5-FU) as positive control. . Cells were exposed to DMSO without sample as negative control. Treatment for the bioactive compound was carried out at different incubation periods (24, 48 and 72 h). Upon completion of incubation, each well was added 20 µL of MTT (5mg/mL) and incubated for 4 h at 37 ºC. All the medium was aspirated and 150 µL of DMSO was added to resuspend the formazan crystals in which measured at 570 nm and 650 nm as reference wavelength by microplate reader. The percentage of cell viability was calculated as followed: Cell viability percentage (%) = (Absorbance of treated cells/absorbance of untreated cells) × 100%.

3.2.17 Effect of DET on nuclear morphological alterations

As mentioned previously in 3.2.5, HCT116 cells were cultured in 60 mm^2 culture dishes and treated with DET in increasing concentrations of 0.75, 1.5 and 3.0 µg/mL or vehicle DMSO (negative control) for 24 h. After treatment, cells were harvested and subjected to

staining procedure accordingly. The nuclear morphological was observed under inverted fluorescence microscopy (Leica DM1600B, Wetzlar, Germany).

3.2.18 Effect of DET on phosphatidylserine externalization

Cells were plated and incubated in presence of DET (0.75, 1.5 and 3.0 µg/mL) for 24 h. Cells were harvested and subjected to staining similarly based on 3.2.6. Then, apoptotic and necrotic cell populations were detected at FL1-A (x-axis) and FL2-A channel (y-axis) by using flow cytometer (Vermes *et al.*, 1995).

3.2.19 Cell cycle assay

Changes in cell cycle distribution was detected according to the manufacturer's procedure. 1×10^6 HCT116 cells were exposed to DET (0.75, 1.5 and 3.0 µg/mL) for 24 h. The harvested cells were centrifuged at 300 xg and washed with 1 mL of buffer solution twice. The cell pellet was resuspended with 250 µL of Solution A (trypsin buffer) for 10 min and 100 µL of Solution B (trypsin inhibitor and RNase buffer) for another 10 min. Subsequently, cells were gently mixed with 200 µL of cold PI solution for 10 min on ice in the dark. The DNA content was assessed by using flow cytometry (BD).

3.2.20 Western blot analysis

As described similarly in chapter 3.2.13, HCT116 cells were incubated with 0.75, 1.5 and 3.0 µg/mL of DET for 24 h. Briefly, the proteins lysates were electrophoresed on the 10 or 12 % SDS-PAGE gels and transferred onto nitrocellulose membranes prior to the blocking with skim milk/ BSA for 1 h. The membranes were probed with primary antibodies overnight at 4°C. After washing, membranes were incubated with corresponding anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Then, the membranes were subjected to the staining by using ECL

detection kit and visualization using gel documentation system followed by quantification with Vilber Lourmart.

3.2.21 Cell Culture and treatment

HCT116 cells were grown in RPMI 1640 medium containing 10% FBS and 0.01% anti-mycotic solution in a humidified atmosphere of 5% CO_2 at 37°C. HCT116 cells were treated with 3.0 µg/mL of DET (purity ≥ 98%) at 6, 12 and 24 h or 0.75, 1.5 and 3.0 µg/mL of DET for 24 h. For the negative control, cells were treated with vehicle DMSO.

3.2.22 Detection of mitochondrial membrane potential

The alteration of mitochondrial membrane potential was investigated by using the mito-ID staining kit. A total density of 1×10^6 HCT116 cells were cultured for 24 h and exposed to 0.75, 1.5 and 3.0 µg/mL of DET. Upon completion of treatment, the cells were harvested and washed. Medium containing mito-ID was added to the cell pellet and incubated for 15 min at 37 °C in 5% CO_2 incubator. The cells were then washed and resuspended in the medium prior to the detection of green and red fluorescence signals in FL1-A and FL2-A channels by flow cytometry.

3.2.23 Assessment of acidic vesicular organelles formation

To detect the occurrence of acidic vesicular organelle (AVO), cells were subjected to acridine orange (AO) staining. 1×10^6 cells were incubated with or without DET in the presence or absence of NAC for 24 h. After treatment, cells were harvested and washed with PBS followed by addition of 1 µg/mL of AO for 15 min at 37 °C. Later, washing step was repeated three times with PBS and visualized by using fluorescence microscopy.

3.2.24 Transmission electron microscopy

To determine the occurrence of autophagosomes induced by DET in HCT116 cells, transmission electron microscopy (TEM) was used to visualize the ultrastructural alterations. Untreated and treated cells were harvested and fixed with 4% glutaraldeyde for 4 h followed by post fixed with 1% osmium tetroxide at 4 ºC for 2 h. The samples were then washed with cacodylate buffer and left overnight in the buffer. The samples were rinsed with distilled water for thrice. This was followed by a series of dehydration process in a gradient of 35, 50, 70% of ethanol (10min each), 95% of ethanol for 15 min, 100% of ethanol thrice for 15 min each and propylene oxide twice for 15 min. Propylene oxide and Epon were mixed in 1:1 ratio and added to samples for 1 h followed by addition of propylene oxide and epon (1:3) to samples to incubate for another 2 h. The cells were then further embedded in epon overnight followed by polymerization at 37 ºC for 5 h and 60 ºC for overnight. Ultrathin sections were obtained by using an ultramicrotome (Leica ultracut, Germany). The sections were placed on the copper grids and stained with uranyl acetate replacement and lead citrate. Subsequently, the sections were visualized under the TEM (Zeiss LEO Libra 120).

3.2.25 Quantification of intracellular reactive oxygen species (ROS) level

The intracellular accumulation of ROS was measured by using fluorescent probe 2'-7'-dichlorofluorescein diacetate (DCFH-DA). The cells were seeded in 60 mm^2 culture dish at a density of 1×10^6 cells and treated with different concentrations of DET, vehicle DMSO or *tert*-butyl hydroperoxide (TBHP) as positive control for 4 h. The cells were THEN rinsed and stained with 50 μM DCFH-DA for 1 h at 37 ºC. Cells were harvested and washed twice with PBS prior to the detection by using flow cytometry at FL1-A channel.

3.2.26 Protein array analysis

A total density of 1×10^6 HCT116 cells were seeded in 60 mm^2 culture dishes followed by exposure to DET (0.75 and 1.5 µg/mL) or vehicle DMSO (negative control) for 24 h. The cells were then harvested and incubated with RIPA lysis buffer. Protein content of lysate was determined by using BCA protein assay and subjected to protein analysis using human apoptosis antibody array (Raybiotech, GA) based on the manufacturer's protocol. The fluorescence intensities were analysed by densitometry.

3.2.27 Western blot analysis

0.75, 1.5 and 3.0 µg/mL of DET or vehicle DMSO were exposed to HCT116 cells for 24 h. Then, the protein lysates were extracted from cells and electrophoresed by 10 or 12 % SDS-PAGE gel followed by the transfer of proteins onto a nitrocellulose membrane and blocking by using skim milk or BSA for 1 h. Incubation of primary antibodies followed by probing with anti-mouse/rabbit secondary antibodies. Subsequently, the membranes were stained by using ECL detection kit and observed under gel imaging system. Band intensity of respective proteins were quantitatively and qualitatively analysed with Vilber Lourmart.

3.2.28 Animals and ethics statement

40 female (BALB/c-nu) nude mice (4 weeks old) were purchased from Invivos Pte. Ltd. Singapore and housed in the Animal Experimental Unit at University Malaya under pathogen-free conditions and supply of sterilized food and water ad libitum. Animals were acclimatized for two weeks and kept in ventilated cages under standard conditions with 12 h light-dark cycle at 24 ºC. Experiments with mice were performed based on the protocol approved by the Faculty of Medicine Institutional Animal Care and Use Committee, University of Malaya with the ethics reference number of 2014-03-

05/IBS/R/CCK. The whole experiment was conducted in the AAALAC International accredited Animal Experimental Unit of the Faculty of Medicine, University of Malaya.

3.2.29 Human colorectal carcinoma xenograft model

On day 0, mice were anaesthetized and HCT116 human colorectal carcinoma cells (1x 10^6) which was resuspended with 50% matrigel (0.1mL) were inoculated subcutaneously into the right flank. When the tumour was palpable (range 100mm^3), six mice were randomly assigned into each group for a total of 4 groups. Tumour size and body weight were recorded prior to the treatment. Mice were intraperitoneally (i.p.) administered with 25 $mLkg^{-1}$ of saline with 4% DMSO or 1.25 mg/kg DET or 2.5 mg/kg DET or 40 mg/kg 5-fluorouracil thrice a week for 28 days. The body weight and tumour growth of each mouse were measured weekly for 4 weeks. The length and width of tumour were measured by using caliper and recorded. The tumour volume was calculated according to the following formula, 0.5 x length x $width^2$. When the tumour nodules reached 1.5 cm in diameter, the mice were sacrificed.

3.2.30 Histology of tumour

Harvested tumour was fixed in 10% buffered formalin and processed by spin tissue processor microm STP 120 (Thermo Scientific) to remove water and solidify the tissue. Samples were embedded with hot paraffin wax using modular tissue embedding center microm EC 350 (Thermo Scientific). Paraffin-embedded tumour were sectioned into 4µm thick tissue by using rotary microtome microm HM 340 E (Thermo Scientific). The sections were then transferred to the poly-L-lysine coated glass slides, left ot dry completely and kept in -20 ºC prior to the immunohistochemical staining.

3.2.31 Immunohistochemical analysis

For immunohistochemical staining, the slides were deparaffinised in three changes of xylene for 2 min each followed by three changes of absolute ethanol for 2 min each and once through 95% ethanol and 80% ethanol for 2 min each. Then, the slides were incubated with 0.3% H_2O_2 for 10 min. Antigen unmasking was carried out by incubating slides in a boil 10mM sodium citrate buffer (pH 6.0) for 30 min. The slides were then incubated with 5% normal goat serum for 1 h at room temperature and probed with primary antibodies overnight at 4 ºC. Prior to the incubation of secondary antibodies for 30 min, washing step was needed to repeat three times with wash buffer (1 x TBST) for 5 min each. The slides were repeatedly washed with TBST three times for 5 min. DAB chromogen was added to the slides and counterstained with haematoxylin. For quantification, three random × 400 microscopic fields per slide were captured using an inverted microscope Nikon Eclipse TS100 (Nikon, Japan) and analysed using Nikon NIS-BR element software (Nikon, Japan).

3.2.32 Cell culture

The mouse microglial BV-2 cells was developed by Dr E. Blasi from University of Perugia which was cultured in DMEM containing 10% FBS and 0.01% anti-mycotic solution and maintained at 37 °C in 5% CO2 incubator.

3.2.33 *In vitro* cell viability assay

The inflammatory effects of ESEAF in LPS-induced BV-2 microglia cells was assessed by MTT assay. A density of 1×10^4 cells per well were plated in a 96-well plate and exposed to ESEAF ranging from 1.56–25 µg/mL for 2 h prior to the treatment of LPS (1 µg/mL) for 24 h. 20 µL of MTT solution (5 mg/mL) was added for 4 h at 37 °C. The cell

viability was analysed by using microplate reader at 570 nm with reference wavelength of 650 nm.

3.2.34 Nitric oxide quantification assay

Quantification of nitric oxide (NO) level was carried out based on the manufacturer's procedure by using Total Nitric Oxide Assay Kit. The NO concentration was determined according to nitrate standard curve. A density of 1 x 10^3 cells which was loaded in a 96-well plate were left to adhere for 24 h followed by incubation of ESEAF (0.13 to 1.0 µg/mL) for 2 h prior to 1.0 µg/mL LPS treatment for 4 h. Subsequently, 50 µL of culture supernatant, 25 µL of NADH and 25 µL of nitrate reductase were mixed and left for 30 min at 37 °C followed by addition of Griess reagents 1 and 2 for another 10 min. Upon completion, the absorbance was scanned by using microplate reader at 570 nm.

3.2.35 Determination of prostaglandin E2 (PGE_2) level

Pre-exposure of ESEAF for 2 h in LPS-stimulated BV-2 cells for 24 h and culture media was harvested to quantify PGE_2 level based on the manufacturer's protocol using PGE_2 Competitive ELISA kit.

3.2.36 Detection of intracellular reactive oxygen species (ROS) level

The production of intracellular ROS level was evaluated by using DCFH-DA probe. 1 x 10^6 cells were treated with ESEAF (0.25, 0.5 and 1.0 µg/mL) for 2 h followed by incubation of LPS for 24 h. Cells were then rinsed and subjected to staining with 10 µM DCFH-DA for 30 min. The DCF fluorescence intensity was detected in FL1-A channel and analysed by flow cytometer.

3.2.37 Determination of scavenging activity by DPPH assay

The effect of ESEAF on scavenging free radical was determined via DPPH assay based on the outlined protocol with minor modifications (Ser *et al.*, 2015). Different concentrations of ESEAF (15.6 µg/mL- 1000 µg/mL) was tested. Addition of 195 µL of 0.016 % DPPH in 95% ethanol to 5 µL of sample solution or gallic acid (positive control) and left in darkness for 30 min and absorbance was measured at 515 nm using a spectrophotometer. The DPPH free radical scavenging activity was calculated as follows: DPPH scavenging activity = $(A_C\text{-As})/A_0$ x 100%, where A_C is the absorbance of control; A is the absorbance of the sample.

3.2.38 Measurement of Superoxide dismutase (SOD) level

Measurement of SOD scavenging activity of ESEAF was performed based on the manufacturer's protocol. 20 µL of ESEAF samples which concentration (0.488 - 31.25 µg/mL) was added to mixture reaction solution containing WST working solution and enzyme working solution for 20 min at 37 ºC followed by the absorbance measurement at 450nm using a microplate reader. The SOD activity of ESEAF (percentage of inhibition %) was calculated using following equation: SOD activity = (A0 –Ab)-(As -Asb) / (A0 – Ab) x 100%, where A0: Absorbance control blank; Ab: Absorbance buffer blank; As: Absorbance sample; Asb: Absorbance sample blank

3.2.39 Catalase assay

Generation of catalase was determined by using catalase assay kit based on the manufacturer's procedure. After exposure of ESEAF to LPS-stimulated BV-2 cells, cells were collected and lysed with lysis buffer. Supernatant were collected and kept for this assay. The sample were then mixed with colorimetric assay substrate solution for 5 min followed by adding stop solution. 10 µL of catalase enzymatic reaction mixture and

colour reagent was left incubation for 15 min prior to the measurement of the absorbance at 520 nm. The catalase activity of ESEAF was evaluated based on the H2O2 standard curve. Catalase activity was calculated as followed: Activity (µmoles/min/mL) = (Δµmoles (H_2O_2) x dilution factor x 100)/ (V x t), where V = sample volume; T = catalase reaction duration (minutes)

3.2.40 Western blot analysis

1 x 10^6 BV-2 cells were subjected to treatment with ESEAF (0.25, 0.5 and 1.0 µg/mL) followed by the treatment of LPS for 24 h. After treatment, adherent and suspended cells were lysed in cold RIPA buffer and further subjected to centrifuge at 14000 rpm for 15 min. The supernatant were obtained and determined protein content. 25 µg of proteins were loaded into each well of 10% or 12% SDS-PAGE which used to separate the protein and transferred onto a nitrocellulose membrane. 5% of skim milk or BSA was used to expose on the membrane for 1 h followed by the incubation of primary antibodies overnight at 4 °C. The membrane was washed thrice for 5 min and then probed with anti-rabbit/mouse secondary antibodies for 1 h at room temperature. The protein on the membrane was stained by using ECL detection kit prior to the visualization under gel documentation system. Each protein band intensity was quantified by using Vilber Lourmart.

3.2.41 Animals

Eight/ten-week-old male C57BL/6 mice (25 ± 2 g) were provided by the Laboratory Animal Centre of the School of Medicine and Health Sciences, Monash University Malaysia. Five mice were kept in a cage under standard conditions with 12 h light-dark cycle and allowed access to pelleted feed and water ad libitum. All the procedures and

experiment was approved by the Animal Ethics Committee from Monash University with the reference number of MARP/2014/022.

3.2.42 Acute oral toxicity assay

Acute oral toxicity study of ESEAF was conducted according to the OECD guideline No 425. Based on the OECD guideline of the fixed dose procedure, each of the fasted mice is administered via oral gavage with a single dose of ESEAF either with the dosage of 5, 50, 300 and 2000 mg/kg. However, a limit test was performed which started with the highest dosage which is 2000 mg/kg of ESEAF. One group is treated with ESEAF with 5 mice while the other 5 mice were treated without ESEAF but sesame oil only (control). The animals were fasted overnight and followed by the treatment. After administration, food was withheld for 3 h. Based on the guideline, one animal was orally administered with 2000 mg/kg of ESEAF which suspended with sesame oil at a time. Subsequently, each mice were sequentially administered at 48 to 72 h intervals. Symptoms of toxicity and mortality were observed daily for a period of 14 days. On the day 0, 7th and 14th, all animals were weighed and recorded. After completed the treatment, all the animals were given 13.5 mg/kg of Zoletil/Ketamine/Xylazine and subjected to undergo cardiac puncture for blood collection followed by collection of organs (kidney, lung, heart and liver).

3.2.43 Preparation of paraffin sections

The collected organs were preserved in a 10% buffered formalin solution. The tissues were processed by spin tissue processor microm STP 120 (Thermo Scientific) to remove water and solidify the tissue. After processing, tissues were orientated inside a mold and embedded with hot paraffin wax using modular tissue embedding center microm EC 350 (Thermo Scientific). Prior to the sectioning, the paraffin blocks are kept in -20 ºC and

subsequently, were sectioned into 4 µm thick tissue by using rotary microtome microm HM 340 E (Thermo Scientific). Sections were left to float on a 40 ºC water bath and transfer onto glass slides. The slides were dried overnight and stored at room temperature.

3.2.44 Histopathological examination

The slides were deparaffinised in three changes of xylene for 2 min each followed by three changes of absolute ethanol for 2 min each and once through 95% ethanol and 80% ethanol for 2 min each. The slides were rinsed with distilled water for 1 min and stained with haematoxylin for 1 min. Later, washing step was repeated before and after the incubation in acid alcohol for 30 sec. The slides were subjected to bluing agent for 30 sec, washed for 1 min and staining with Eosin Y for 30 sec. 95% ethanol and two changes of absolute ethanol was used to rinse for 1 min following by xylene for 1 min. Slides were mounted by using mounting media and ready for observation under the microscope.

3.2.45 Blood biochemical analysis

Collected blood samples were immediately centrifuged at 3000 rpm for 15 min and supernatant was aliquoted as serum. Blood serum obtained were assessed for the biochemical parameters such as albumin, total bilirubin levels (TBIL), alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and creatinine (CRE).

3.2.46 Statistical analysis

In all the experiments, statistical analysis was conducted by using one-way ANOVA followed by Dunnett's test or Student's *t*-test to compare significance between untreated control group and treated groups. All data were expressed as means ± standard. Data was regarded as statistically significant when p values <0.05.

CHAPTER 4: RESULTS

4.1 The extract and fractions of *E. scaber* inhibit the cancer cell growth

All the *E. scaber* crude and fractions were subjected to assess cytotoxic activities on different cell lines including HT-29 human colon adenocarcinoma cells, HCT116 human colorectal carcinoma cells and CCD841-CoN normal colon cells via MTT assay. Treatment with varying concentrations of ESEE, ESHF, ESEAF and ESAF (0.1953 – 200 µg/mL) were exposed to all the cells. ESEAF demonstrated the greatest growth inhibitory effect among all the fractions on both HCT116 and HT-29 cancer cells at 72 h, followed by ESHF, ESEE and ESAF (Figure 4.1 (a)&(b)). However, a remarkable reduction of HCT116 cell viability was observed upon exposure to ESEAF at 72 h with the lowest IC_{50} values of 1.42 ± 0.10 µg/mL indicating its most remarkable potency towards HCT116 cells (Figure 4.1). All the IC_{50} value were shown and encapsulated in Table 4.1. In order to investigate any toxic effect of ESEAF on human normal cells, human normal colon CCD-841CoN cells was used. As shown in Figure 4.1 (c), normal colon cells was remained viable at the highest concentration of 25 µg/mL. According to the results, ESEAF and HCT116 cells were proceeded for further investigation underlying its apoptosis-inducing effects.

4.2 ESEAF induced nuclear morphological alterations

Hoechst 33342 and Propidium Iodide (PI) staining was used to detect the alterations of nuclear morphology in HCT116 cells when treated with ESEAF and visualized under fluorescence microscope. Healthy and viable cells were displayed in dull blue colour. Conversely, early and late apoptotic cells were displayed in bright blue colour and purple colour which dual-stained with bright blue and red colour, respectively. For dead cells, it was displayed in red colour. As shown in Figure 3.2, different apoptotic morphological characteristics including nuclear shrinkage, chromatin condensation and

Table 4.1: IC_{50} values of ethyl acetate fraction of *Elephantopus scaber* leaves and 5-fluorouracil against HCT116 and HT-29 cells and normal colon cells

Cell lines	IC_{50} (µg/mL)				
	Ethanol Extract (ESEE)	Hexane Fraction (ESHF)	Ethyl acetate fraction (ESEAF)	Aqueous Fraction (ESAF)	5-fluorouracil*
HCT116	75.36 ± 0.66	6.65 ± 0.19	1.42 ± 0.08	>200	0.73 ± 0.02
HT-29	134.10 ± 1.64	18.25 ± 0.93	2.41 ± 0.26	>200	NA
CCD-841CoN	NA	NA	>25	NA	>25

The data represent mean ± S.E. of three independent experiments (n = 9). NA: Not available.

* 5-Fluorouracil served as positive control.

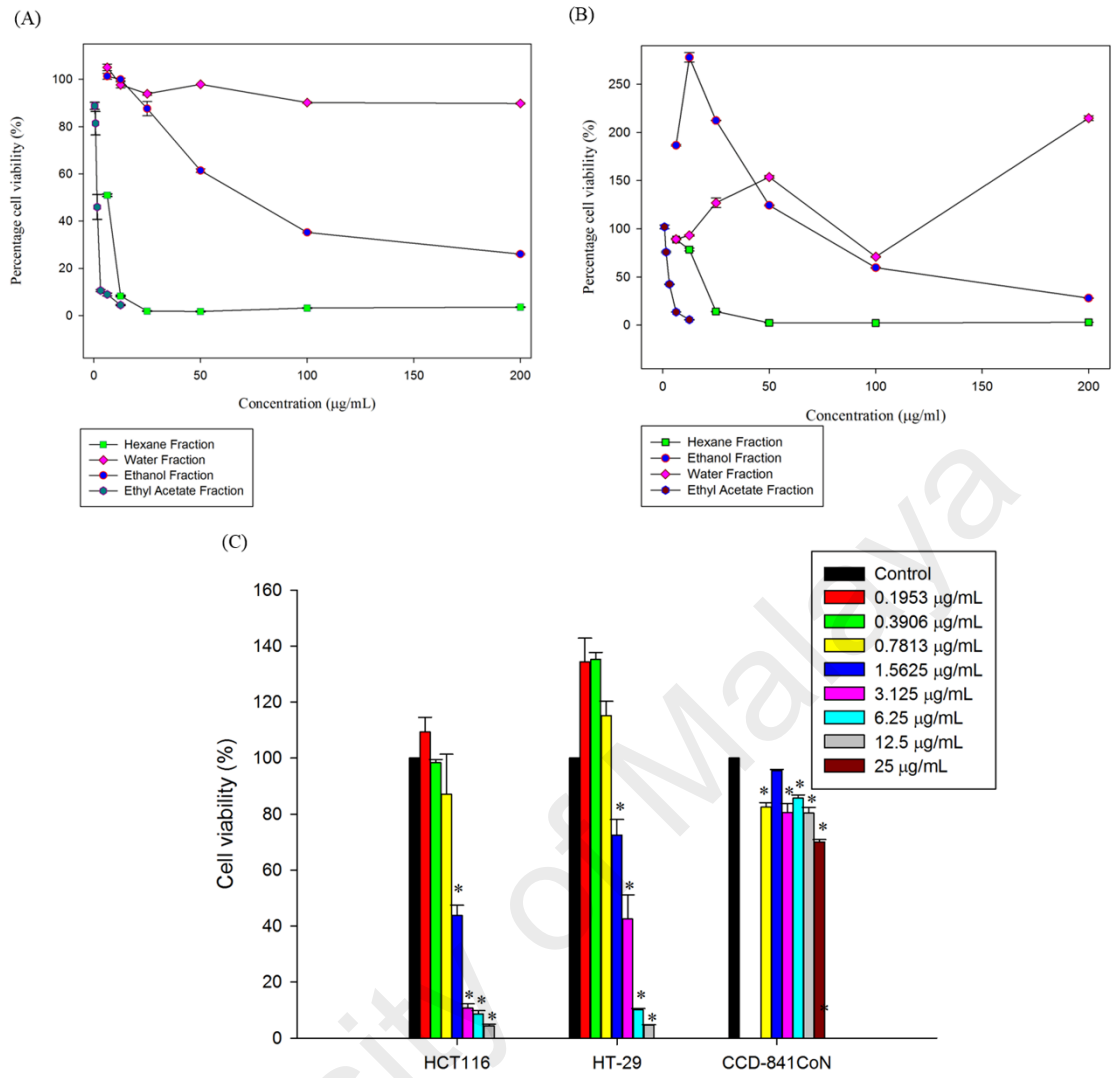


Figure 4.1: The cytotoxicity of *E. scaber* leaves ethanol extract, hexane, ethyl acetate and aqueous fractions against different cancer cell lines and normal cell line. The cells were exposed to different concentrations of extract and fractions of *E. scaber* for 72 h. (A) The graph represented the cytotoxicity against HCT116 cells (B) The graph represented the cytotoxicity against HT-29 cells (C) Bar chart showed the cytotoxic effect of ESEAF against HCT116, HT-29 and normal CCD841CoN colon cells at 72 h. The data represented as mean ± S.E. of three independent experiments (n = 9). Asterisks represent significantly different value when compared to control (* $p < 0.05$).

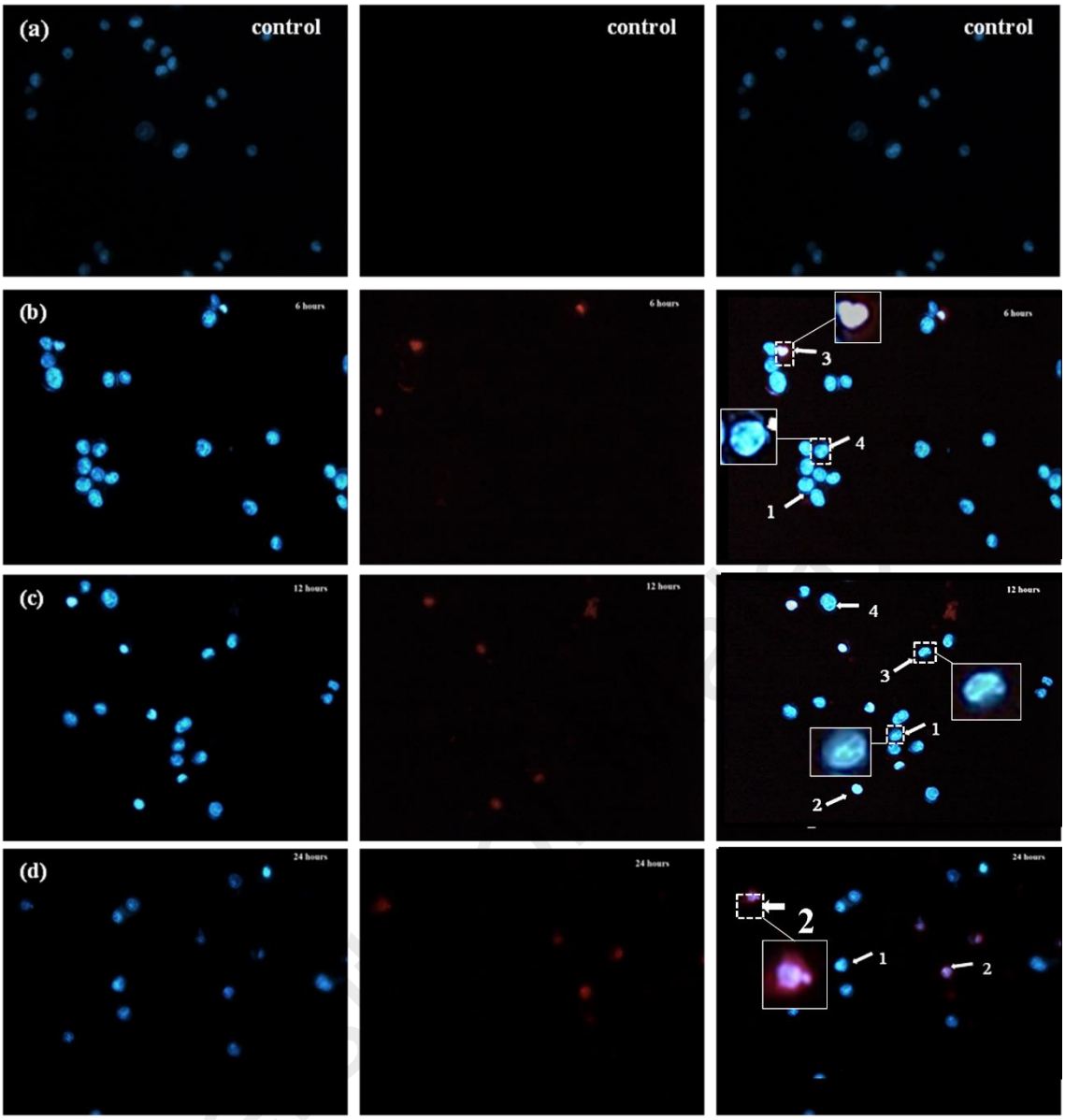


Figure 4.2: Alteration of nuclear morphology by ESEAF in HCT116 cells. Cells were exposed to ESEAF in different incubation periods and subjected to Hoechst 33342 and PI staining followed by visualization using fluorescence microscope. Magnification: 400X Arrow 1 chromatin condensation, 2 late apoptosis/ necrosis, 3 cell shrinkage, 4 DNA fragmentation

DNA fragmentation were detected upon treatment with ESEAF in HCT116 cells. The appearance of the apoptotic cells became more apparent in increasing incubation period.

4.3 ESEAF induced DNA breakage

DNA fragmentation which considered as one of late apoptotic events can be determined by TUNEL assay. The results revealed that there is an elevation of the percentage of TUNEL-positive cells upon exposure to 10 µg/mL of ESEAF (Figure 4.3(a)) in which increased to 1.13 ± 0.09, 1.33 ± 0.07 and 3.09 ± 0.03% at 6, 12 and 24 h, respectively as compared to the untreated group (Figure 4.3(b)). These data suggested that induction of DNA fragmentation was mediated by ESEAF in HCT116 cells.

4.4 The effect of ESEAF on phosphatidylserine externalization

One of the early apoptosis characteristics exhibited is the externalization of phosphatidylserine which can be examined by using Annexin V-FITC/ PI staining. As shown in Figure 4.4(a) & (d), a significant dose-dependent and time-dependent manner elevation in phosphatidylserine externalization upon treatment with ESEAF in HCT116 cells. Figure 4.4 ((b) & (e)) demonstrated the distribution of the viable, early apoptotic, late apoptotic and necrotic cells. The untreated control cells exhibited weak Annexin V and PI staining suggesting viable cells. When treatment with different concentrations of ESEAF, the viable HCT116 cells gradually progressed to early and late apoptosis. The total Annexin V-positive cells ($Annexin^+/PI^-$ and $Annexin^+/PI^+$) which composed of early and late apoptotic cell populations markedly elevated to 4.64 ± 0.12, 8.14 ± 0.47, 16.39 ± 1.28 at concentrations of ESEAF 2.5, 5.0 and 10.0 µg/mL, respectively as shown in Figure 4.4(c). In addition, the percentages of total Annexin V-positive cells increased to 1.77 ± 0.03, 3.62 ± 0.30 and 16.39 ± 1.28 at 6, 12 and 24 h, respectively (Figure 4.4(f)).

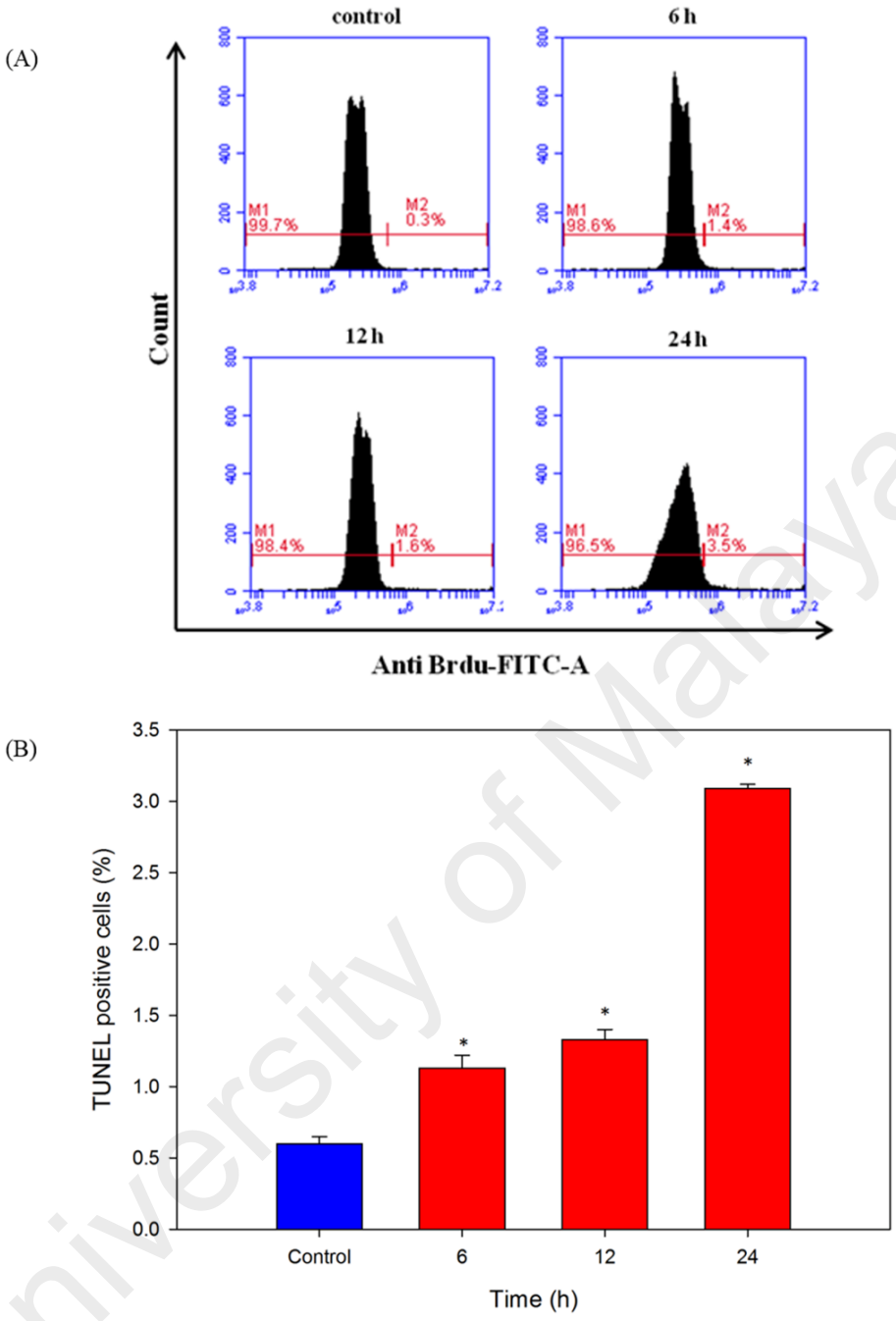


Figure 4.3: Induction of DNA fragmentation in HCT116 cells. Exposure of 10 µg/mL of ESEAF in HCT116 cells for different incubation periods (6, 12 and 24 h) and proceeded to TUNEL staining (A) Histogram depicted positive TUNEL staining which indicated by the M2 region. (B) Bar chart demonstrated the percentage of TUNEL positive cells. Data represented in mean ± S.E. of three separate experiments (n=3). Asterisks denote significant different value in comparison of control (* $p < 0.05$).

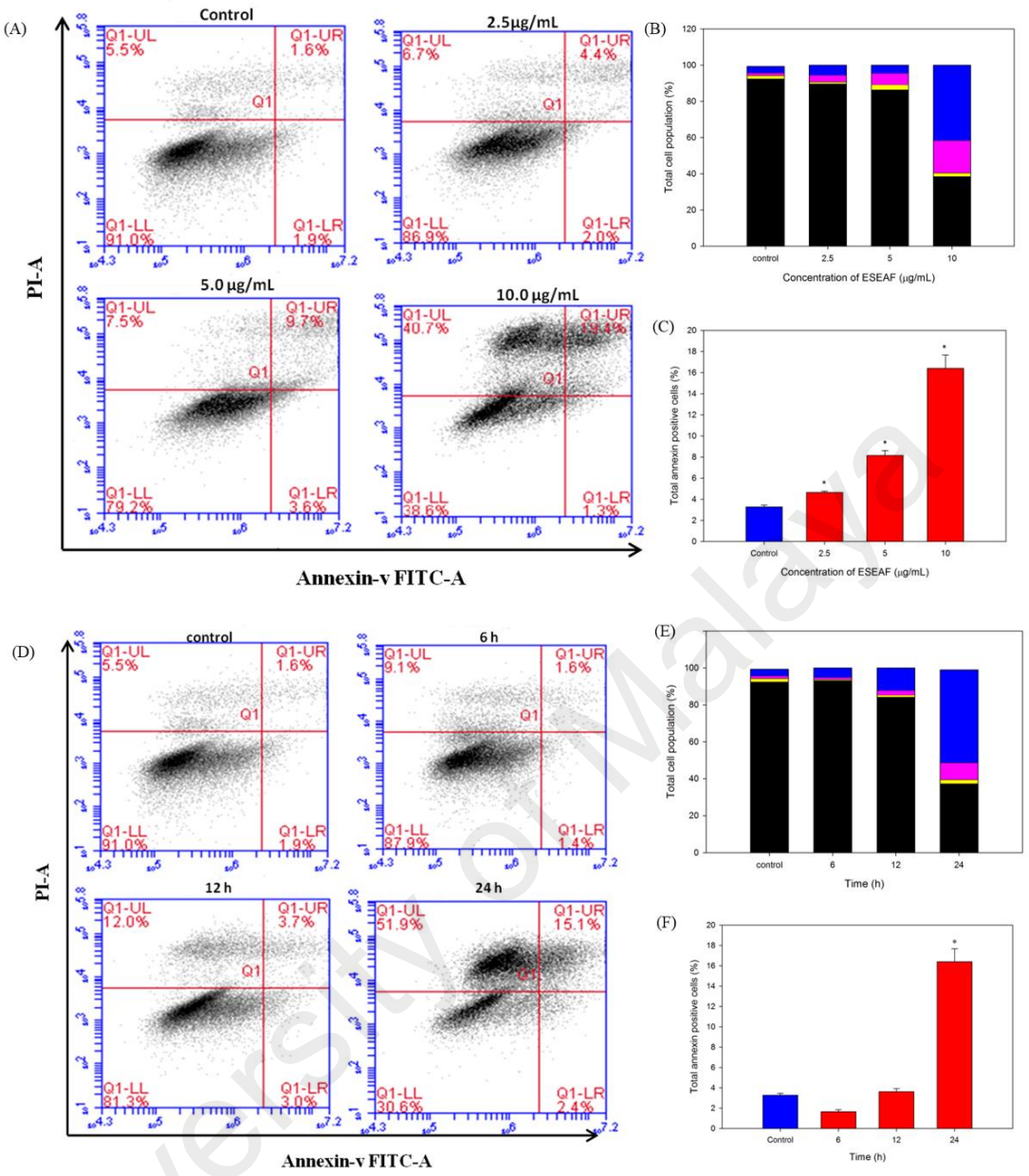


Figure 4.4: Externalization of phosphatidylserine induced by ESEAF in HCT116 cells. (A) Dot plots exhibited the fluorescence patterns of Annexin V-FITC/PI staining upon treatment with different doses of ESEAF at 24 h. (B) Bar chart illustrated the proportion of viable, early apoptotic, late apoptotic and necrotic cells at increasing concentrations of ESEAF. (C) Bar chart demonstrated the percentage of Annexin V positive cells. (D) Dot plot illustrated the fluorescence patterns of Annexin V-FITC/PI staining after exposure of 10.0 µg/mL of ESEAF at 6, 12 and 24 h. (E) Bar chart depicted the proportion of viable, early apoptotic, late apoptotic and necrotic cells at 6, 12 and 24 h. (F) Bar chart illustrated the percentage of Annexin V positive cells in a time course study. The data represented as mean ± S.E. from three individual experiments (n=3). Asterisks denote significantly different value from control (*p<0.001).

4.5 Formation of Intracellular Reactive Oxygen Species (ROS) induced by ESEAF

In order to detect the effect of ESEAF in triggering the production of intracellular ROS in HCT116 cells, DCFH-DA dye was used and Ter-Butyl hydroperoxide was utilized as positive control. As shown in Figure 4.5(a), the right shift of the histogram was observed depicting the increase of intracellular ROS. The intracellular ROS level clearly enhanced by 1.38 ± 0.05, 1.85 ± 0.11, 2.13 ± 0.08, 3.84 ± 0.17 folds, respectively at 1.25, 2.5, 5.0 and 10.0 µg/mL of ESEAF (Figure 4.5(b)). Furthermore, to investigate whether apoptosis was initiated in response to the oxidative stress stimuli, ROS, N-acetylcysteine (NAC) inhibitor was utilised. Results demonstrated that the accumulation of intracellular ROS level was reversed by the pretreatment of NAC in ESEAF-treated cells (Figure 4.5(c)). Additionally, the cell viability was assessed upon the presence and absence of NAC in the ESEAF-treated and untreated cells at 24 h. In presence of NAC, the percentage of cell viability was restored in the ESEAF-treated cells as compared to the absence of NAC (Figure 4.5(d)).

4.6 ESEAF induced loss of mitochondrial membrane potential

The dissipation of mitochondrial membrane potential can be observed during early apoptosis in intrinsic pathway. Therefore, JC-1 probe was applied to investigate the loss of mitochondrial membrane potential in ESEAF-treated HCT116 cells. When mitochondria depolarized in the early apoptosis, the red fluorescent JC-1 aggregates convert to green JC-1 monomers. The untreated cells emerged to have the highest accumulation of red JC-1 aggregates in the mitochondrial membrane (Figure 4.6(a) & (b)). However, the emergence of green monomer fluorescence accompanied by the loss of red JC-1 aggregate fluorescence was observed at 6 h in the treated cells. At 24 h of treatment, the red fluorescence was almost completely absent in HCT116 cells. ESEAF

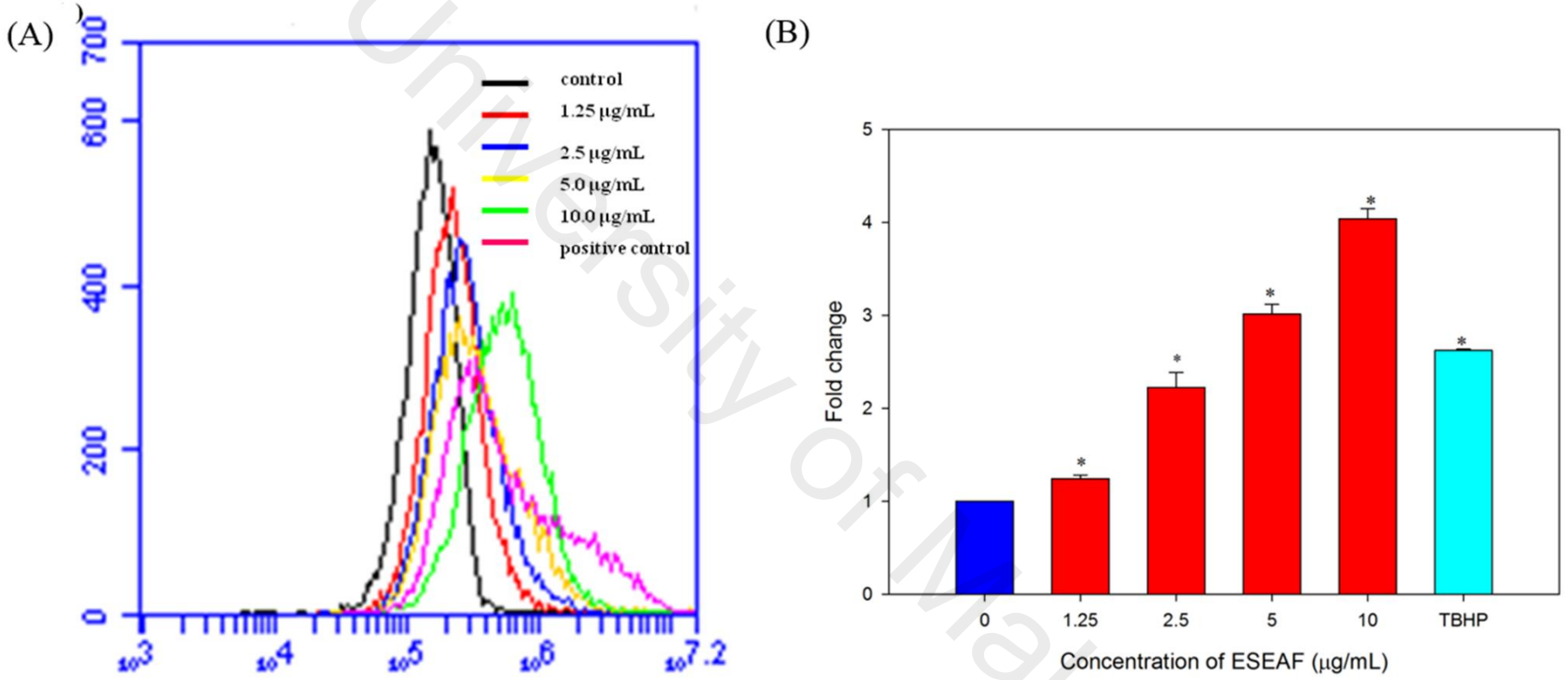


Figure 4.5: The intracellular ROS level in ESEAF-treated cells. (A) Histogram depicted the generation of ROS in response to 2.5, 5.0 and 10.0 µg/mL of ESEAF. (B) Bar chart displayed the fold change of the intracellular ROS level.

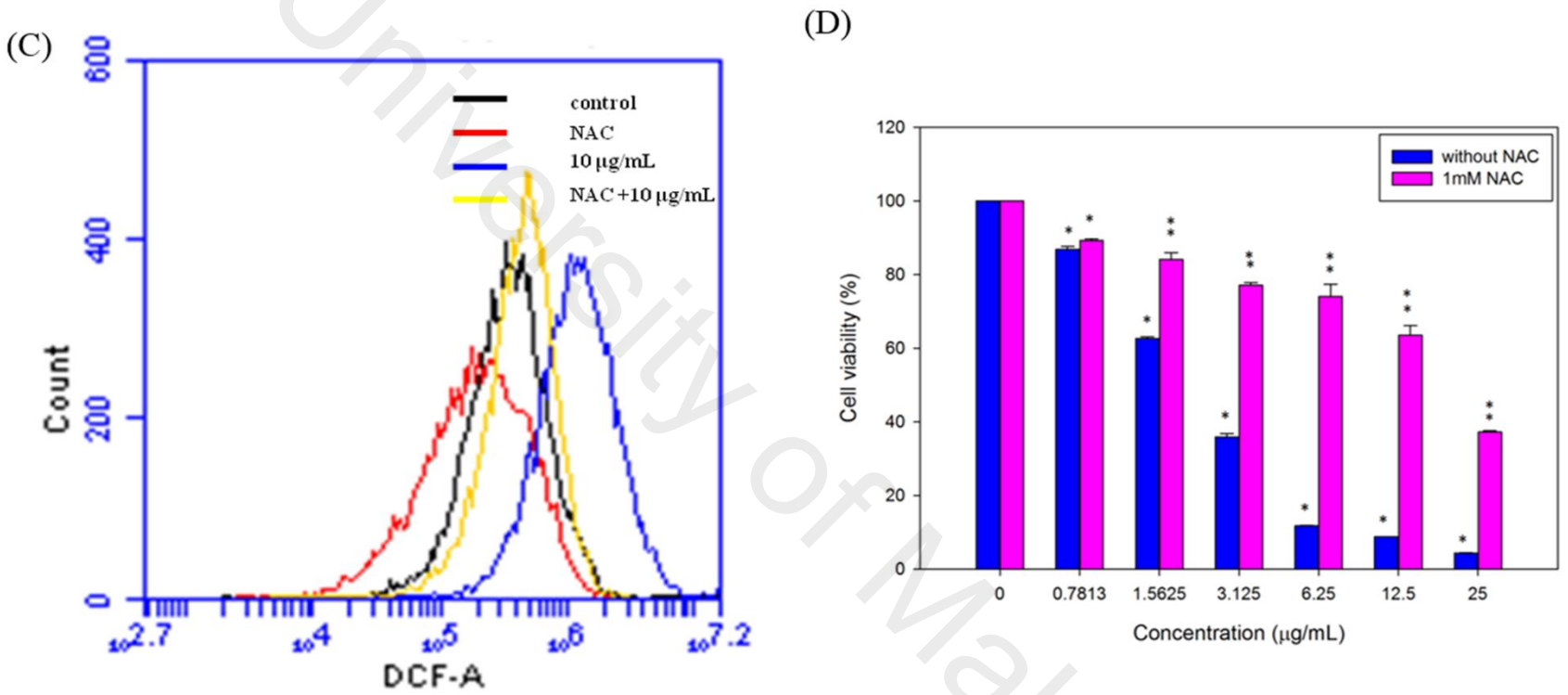


Figure 4.5, continued. (C) Histogram revealed the formation of ROS in the presence and absence of 1mM NAC in the ESEAF treated cells. (D) Bar chart represented the cell viability of HCT116 when pre-incubated with and without 1mM NAC for 1 h prior to the ESEAF treatment (25-200 µg/mL) for 24 h via MTT assay. The data represented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value when compared to control (*p<0.001). Double asterisks denote significantly different value when compared to the treated-cells without

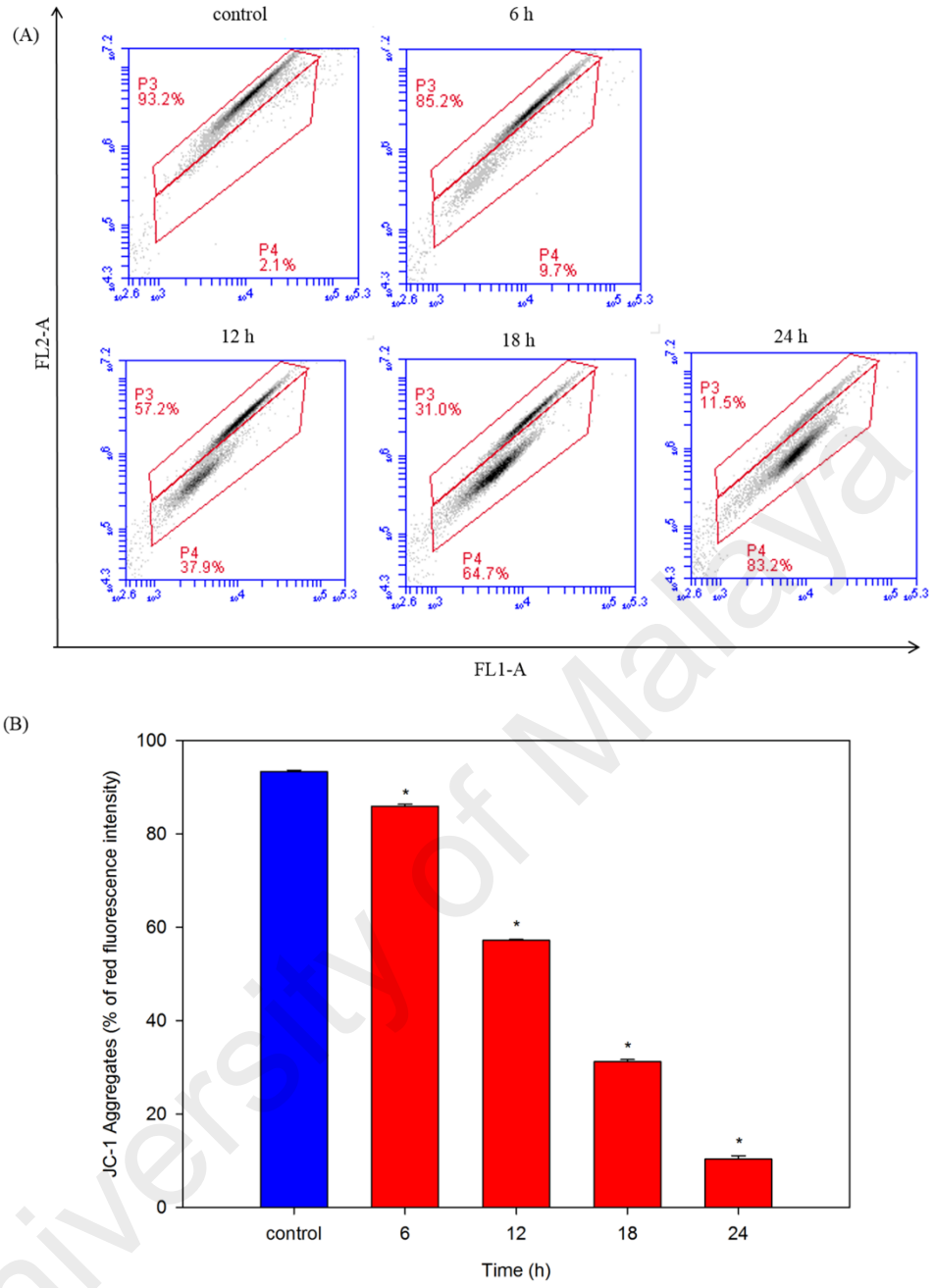


Figure 4.6: Dissipation of mitochondrial membrane potential upon treatment with ESEAF in HCT116 cells. (A) Dot plots of JC-1 aggregates versus JC-1 monomers. (B) The bar chart demonstrated the time-dependent reduction of JC-1 aggregates. The data represented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value when compared to control (*p <0.001).

was found to markedly reduce the mitochondrial membrane potential of HCT116 cells in a time-dependent manner.

4.7 ESEAF modulated the protein expression of Bax and Bcl-2

Proapoptotic Bcl-2 member such as Bax works in concerted with antiapoptotic Bcl-2 member such as Bcl-2 regulates mitochondrial integrity. To investigate the effects of ESEAF on the regulation of the Bcl-2 family members, the expression of Bcl-2 and Bax were examined using flow cytometry analysis. As shown in Figure 4.7(a) & (c), ESEAF induced a time-dependently upregulation of Bax which increased by 1.31 and 1.8 fold at 6 and 12 h, respectively. On the other hand, Bcl-2 expression was slightly increased when treated with ESEAF (Figure 4.7(b) & (d)). However, the ratio of Bax/Bcl-2 was significantly increased by 1.34 folds at 12 h by the elevation of Bax protein expression (Figure 4.7(d)) delineating ESEAF mediated apoptosis via modulation of Bcl-2 and Bax in HCT116 cells.

4.8 ESEAF induced caspase cascades (caspase-3/7 and caspase-9)

Caspases play elemental roles in executing the apoptosis machineries. Dysregulation of Bcl-2 family members led to the release of apoptotic substrates followed by activation of caspase cascades prior to the execution of apoptosis. To examine the involvement caspase-3/7 and -9 instigated by ESEAF, caspase-3/7 (FAM-DEVD-FMK) and caspase-9 (FAM-LEHD-FMK) inhibitors were used. The intensity of fluorescence is directly proportional with the caspase activities. In the results, caspase-3/7 and caspase-9 activities were found time-dependently enhanced when exposure to ESEAF in HCT116 cells (Figure 4.8). It is evidenced by the right shift of histogram representing the elevation of fluorescence intensity. ESEAF activated caspase-3/7 by 2.16, 5.25 and 6.81 folds at 6, 12 and 18 h. Similarly, ESEAF remarkably induced caspase-9 activation by 1.35 fold as early as 6 h followed by increment of 4.67 and 7.23 fold at 12

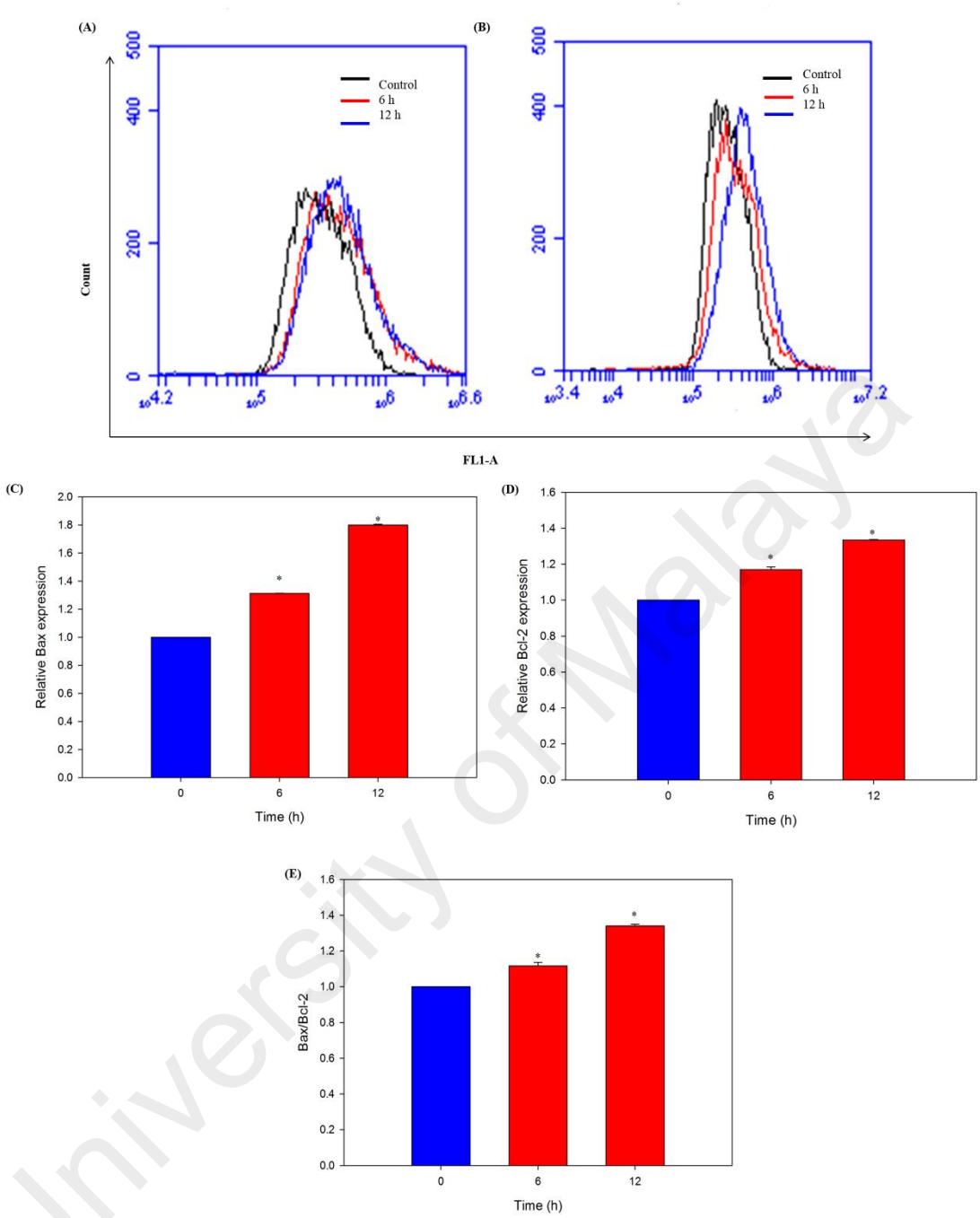


Figure 4.7: The effect of ESEAF on the involvement of Bax and Bcl-2. (A) The histogram displayed the Bax protein expression in the ESEAF-treated cells at 6 and 12 h. (B) The histogram displayed the Bcl-2 protein expression in the ESEAF-treated cells at 6 and 12 h. (C) Bar chart depicted the relative expression level of Bax protein. (D) Bar chart exhibited the relative expression level of Bcl-2 protein. (E) Bar chart represented the ratio of Bax/Bcl-2. The data represented as mean ±S.E. from three individual experiments. Asterisks denote significantly different value when compared to control (* $p < 0.001$).

and 18 h, respectively. Accordingly, the collective results suggested that caspase-3/7 and caspase-9 involved in the induction of apoptosis conferred on ESEAF-treated HCT116 cells.

4.9 The activation of p53 by ESEAF

The deletion or mutation of the p53 gene is the most frequent alteration in human colorectal cancer. Mutation of a tumour suppressor, p53, leads to less susceptible to apoptotic signal. Nonetheless, activation of p53 can be initiated in response to redox balance and DNA damage. As depicted in Figure 4.9, the induction of p53 began as early as 6 h and significantly enhanced at 24 h as demonstrated by the right shift of histogram representing an elevation of fluorescence intensity (Figure 4.9(a)). Treatment of ESEAF resulted in an increase of p53 activation by 1.10, 1.52 and 2.41 folds at 6, 12 and 24 h, respectively (Figure 4.9(b)).

4.10 Induction of PARP cleavage by ESEAF

Caspase cascades facilitates degradation of PARP which consequently leads to DNA fragmentation and ultimately apoptosis. Therefore, the effect of ESEAF on the cleavage of PARP was evaluated by using western blot analysis. A dose-dependent accumulation of cleaved PARP was noticed in HCT116 cells upon treatment with ESEAF as shown in Figure 4.10(a). The protein expression of cleaved PARP was drastically augmented by 1.25 ± 0.02, 1.62 ± 0.08, 4.37 ± 0.28 folds at 2.5, 5.0 and 10.0 μg/mL of ESEAF at 24 h (Figure 4.10(b)). Next, NAC was incubated prior to the incubation with ESEAF to investigate the involvement of ROS in apoptosis induction in HCT116 cells. The presence of NAC attenuated the cleavage of PARP and reversed the apoptosis in ESEAF-treated cells (Figure 4.10(c)). These results suggested the cleavage of PARP was stimulated upon oxidative stress which ultimately resulted in apoptosis.

4.11 Phytochemical profile of ESEAF using GC–MS analysis

As illustrated in Figure 4.11, phytochemical profiling of ESEAF was detected by using GC-MS-TOF. The chromatogram revealed that eight different compounds were discovered in ESEAF (Table 4.2). Several of these compounds were reported to possess anticancer potentials.

4.12 Elucidation of the bioactive compounds from *E. scaber* via bioassay-guided approach

In our previous findings, ESEAF was found to be the most cytotoxic against HCT116 cells. Therefore, bioassay-guided approach was performed to identify and isolate bioactive compounds as shown in Figure 4.12. Two compounds, compound 1 and compound 2 were discovered from active fraction F2 and further proceeded to structural identification by using spectroscopic analysis (^{1}H, ^{13}C NMR and HRESI-MS). The results obtained were compared according to the literature spectral data obtained. Based on the results obtained, compound 1 was elucidated as isodeoxyelephantopin (isoDET) in which 1H and 13C NMR data of were corresponded with the values of isoDET (Paul Pui-Hay But, 1997), as encapsulated in Table 4.3. HRESI-MS, in a positive mode, further validated the structure of compound 1 as isoDET with a molecular formula of $C_{19}H_{20}O_6$ evidenced by the molecular ion peak at m/z 345.1339 $[M + H]^+$. Further on, compound 2 was confirmed as deoxyelephantopin (DET) which found in parallel with the literature values of ^{1}H and ^{13}C NMR data of DET as shown in Table 4.4 (Ni Ni Thana & Heinz H. Fiebig, 2005). The structure of compound 2 which has a molecular formula of $C_{19}H_{20}O_6$ was further supported by HRESI-MS, in a positive mode revealing the molecular ion peak at m/z 345.1332 $[M + H]^+$.

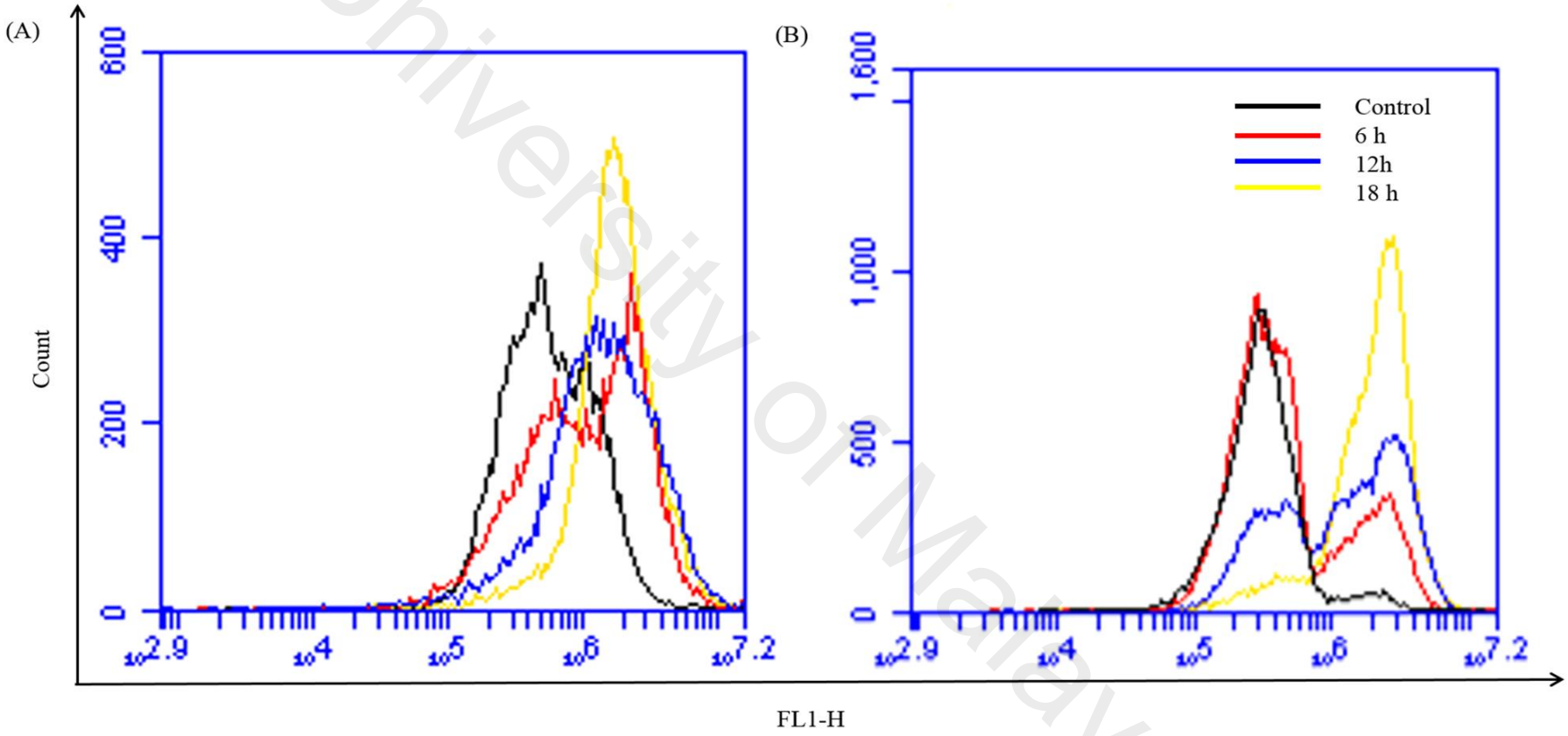


Figure 4.8: Activation of caspase cascades elicited by ESEAF in HCT116 cells. (A) Representative histogram illustrated the flow cytometric fluorescence patterns of caspase-3/7. (B) Representative histogram illustrated the flow cytometric fluorescence patterns of caspase-9.

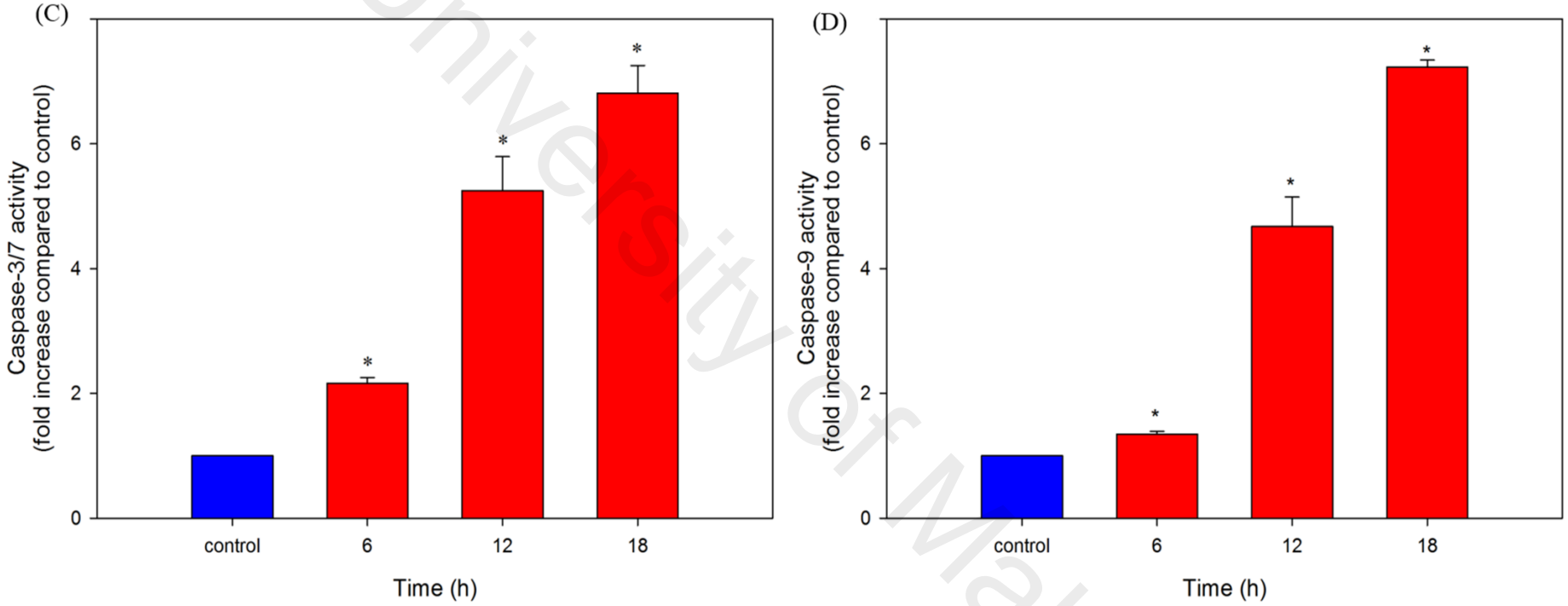


Figure 4.8, continued (C) Bar chart depicted the activity of caspase- 3/7 in ESEAF-treated HCT116 cells. (D) Bar chart depicted the activity of caspase-9 in ESEAF-treated HCT116 cells. Caspase activities were examined upon treatment of ESEAF (10 µg/mL) at 6, 12 and 18 h. The data represented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value in comparison to control (*p< 0.001).

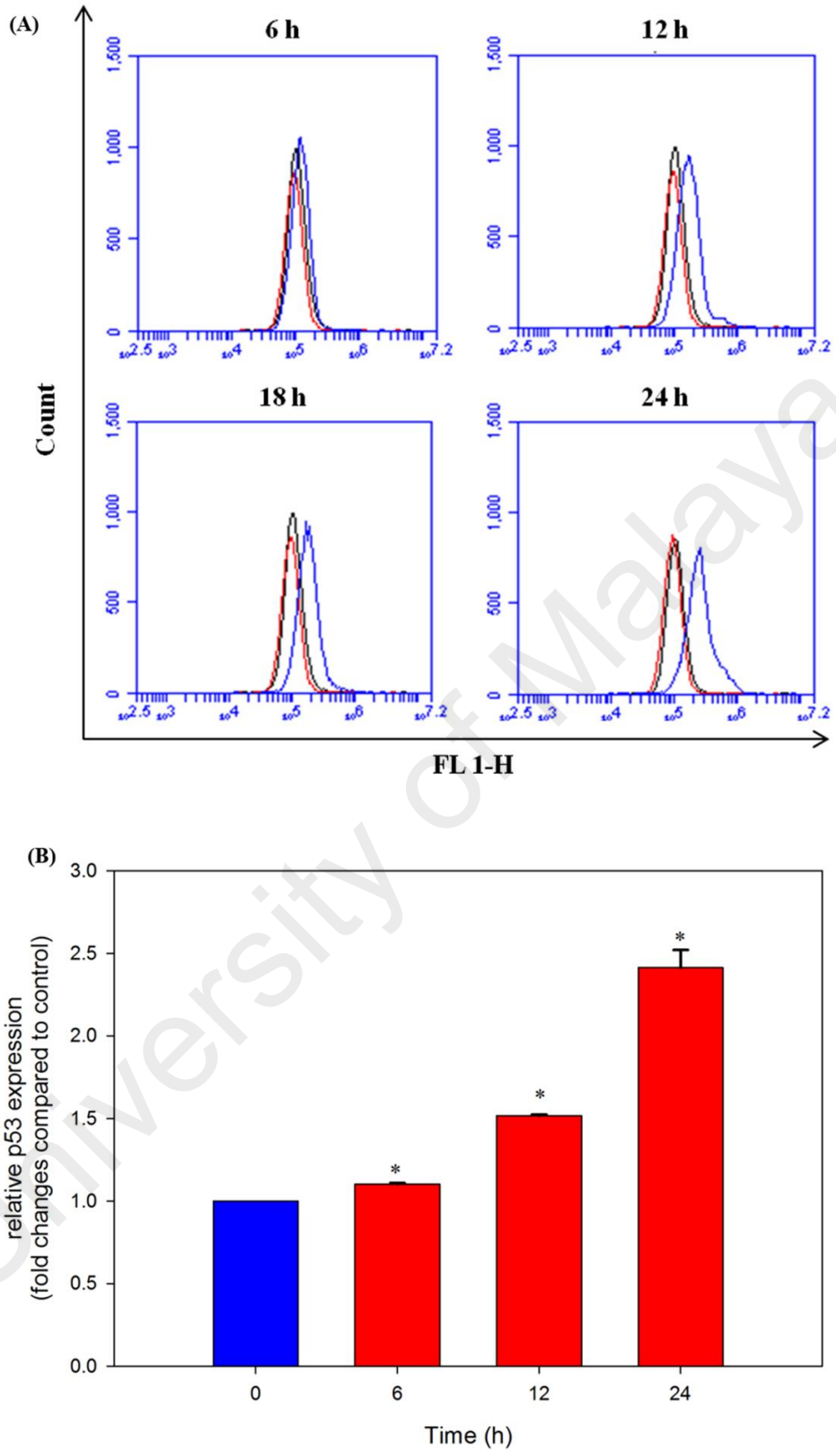


Figure 4.9: The activation of p53 by ESEAF in HCT116 cells. (A) The histogram represented the p53 expression in HCT116 cells at 6, 12 and 24 h. (B) The bar chart illustrated the relative expression level of p53. The data represented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value when compared to control (*p< 0.001).

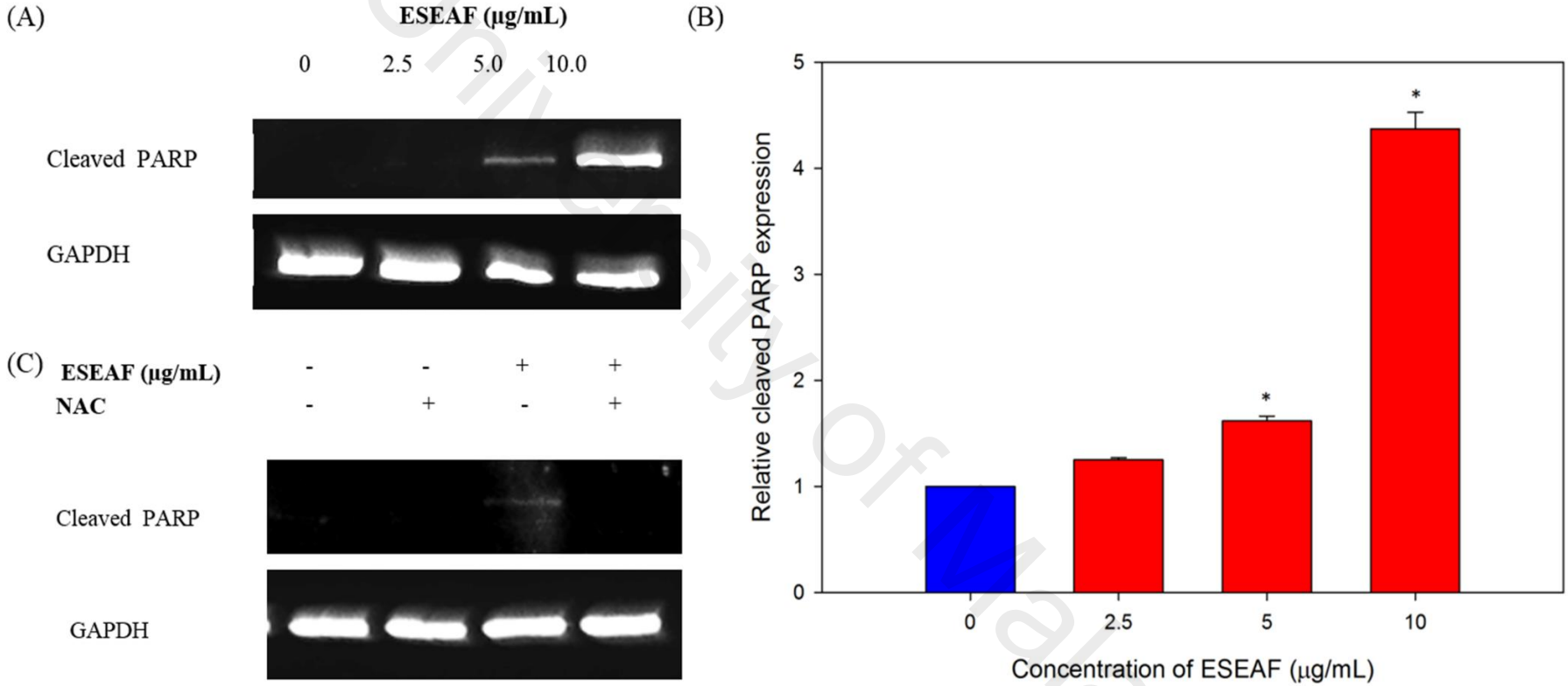


Figure 4.10: Cleavage of PARP in ESEAF-treated cells. (A) The representative western blot band intensity image of cleaved PARP. (B) The bar chart displayed the relative expression of cleaved PARP when exposure to ESEAF (2.5-10.0 µg/mL) in HCT116 cells. (C) The expression of cleaved PARP in presence of 1 mM of NAC in ESEAF-treated cells. GAPDH as a normalization control. The data represented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value in comparison to control (*$p < 0.05$).

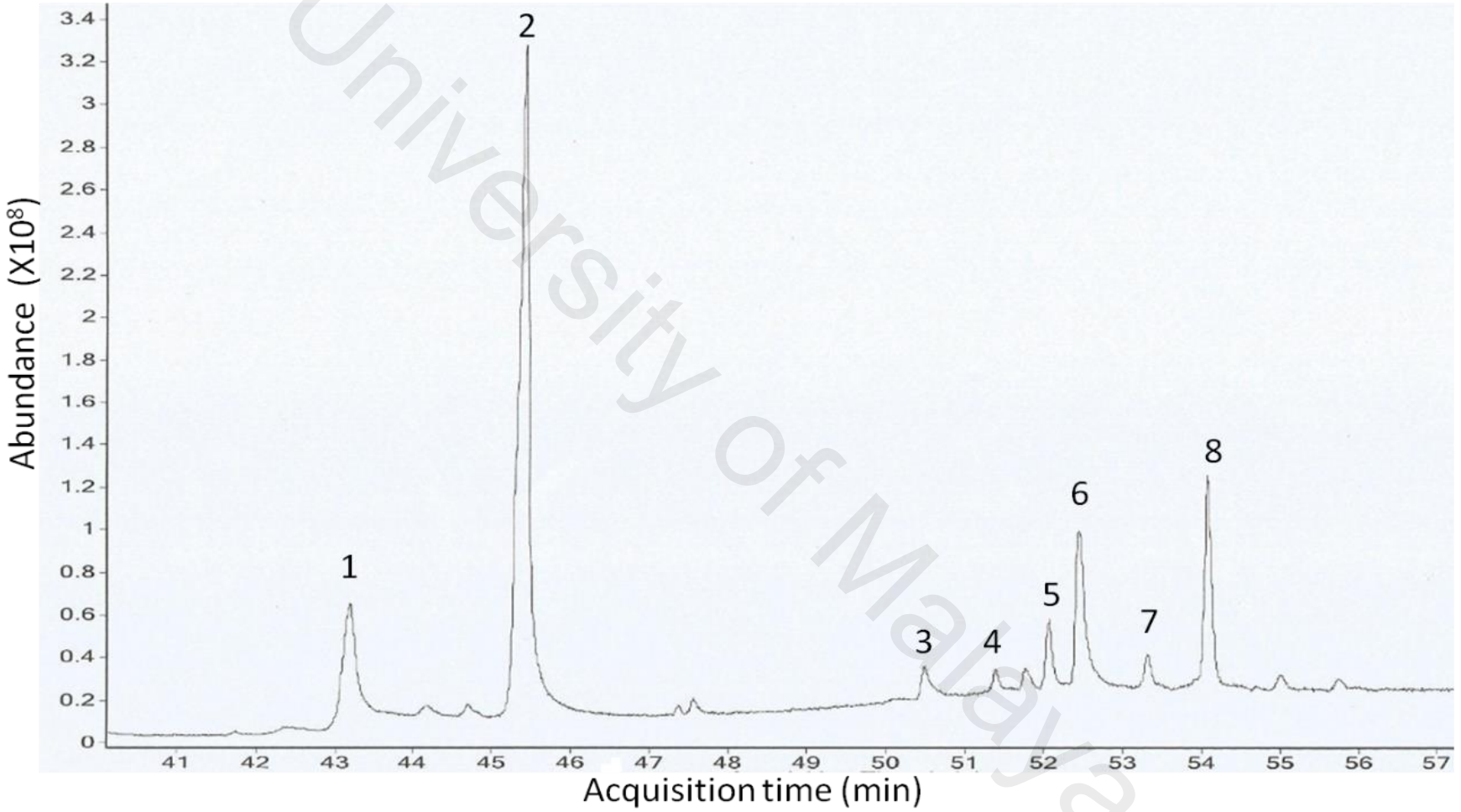


Figure 4.11: GC-MS analysis of ESEAF.

Table 4.2: Chemical constituents identified in ESEAF by using GC-MS

No	Compounds	M.W.	M.F.	Retention Time (s)
1.	Deoxyelephantophin	344	$C_{19}H_{20}O_6$	43.196
2.	Isodeoxyelephantopin	344	$C_{19}H_{20}O_6$	45.436
3.	Stigmasterol	412	$C_{29}H_{48}O$	50.494
4.	Olean-12-en-3-one	424	$C_{30}H_{48}O$	51.403
5.	Lup-20(29)-en-3-one	424	$C_{30}H_{48}O$	52.060
6.	Lupeol	426	$C_{30}H_{50}O_9$	52.457
7.	Betulin	442	$C_{30}H_{50}O_2$	53.324
8.	Lup-20(29)-en-3-ol, acetate, (3β)-	468	$C_{32}H_{52}O_2$	54.083

M.W.: molecular weight; M.F.: molecular formula

4.13 DET and isoDET suppressed the growth proliferation on HCT116 cells

Bioactive compounds, DET and isoDET were subjected to cytotoxic evaluation by using MTT assay on HCT116 human colorectal carcinoma cells and CCD841CoN normal colon cells. As shown in Figure 4.13(a), DET and isoDET were found to dose-dependently reduce cell proliferation of HCT116 at 72 h with IC_{50} values of 0.73 ± 0.01 µg/mL (2.12 µM) and 0.88 ± 0.02 µg/mL (2.56 µM), respectively (Table 4.5). Besides, DET was found to be less potent in CCD841CoN normal cells with an IC_{50} value of 21.69 ± 0.92 µg/mL (60.02 µM). Based on the comparison between IC_{50} values of HCT116 cells and CCD841CoN normal cells, results demonstrated a 30 fold difference in cytotoxicity. Surprisingly, 5- fluorouracil displayed a comparable IC_{50} value of 0.73 ± 0.02 µg/mL (5.0 µM) in HCT116 cells. In addition, exposure to DET resulted in a time-dependent inhibition on the HCT116 cell growth with the IC_{50} values of 2.36 ± 0.02, 0.9 ± 0.02 and 0.73 ± 0.01 µg/mL at 24, 48 and 72 h, respectively (Figure 4.13(b)).

4.14 Alterations of nuclear morphology by DET

Hoechst 33342/PI staining was utilized to detect the alterations of nuclear morphology after exposure of DET in HCT116 cells. Upon treatment with DET, apoptotic morphological changes of HCT116 cells including chromatin condensation, cell shrinkage, DNA fragmentation and apoptotic bodies which were either stained in bright blue or bright blue and red color were detected under fluorescence microscope (Figure 4.14). Treated cells emerged in round shape and homogenously stained with intact chromatin in the nuclei. Apoptotic cells and necrotic cells clearly increased concomitantly with a dose-dependent decrease in cell viability by DET at 24 h.

4.15 Effect of DET on externalization of phosphatidylserine

Next, the biochemical changes resulting in apoptosis-inducing effect by DET was investigated in HCT116 cells. Externalization of phosphatidylserine is characterized as

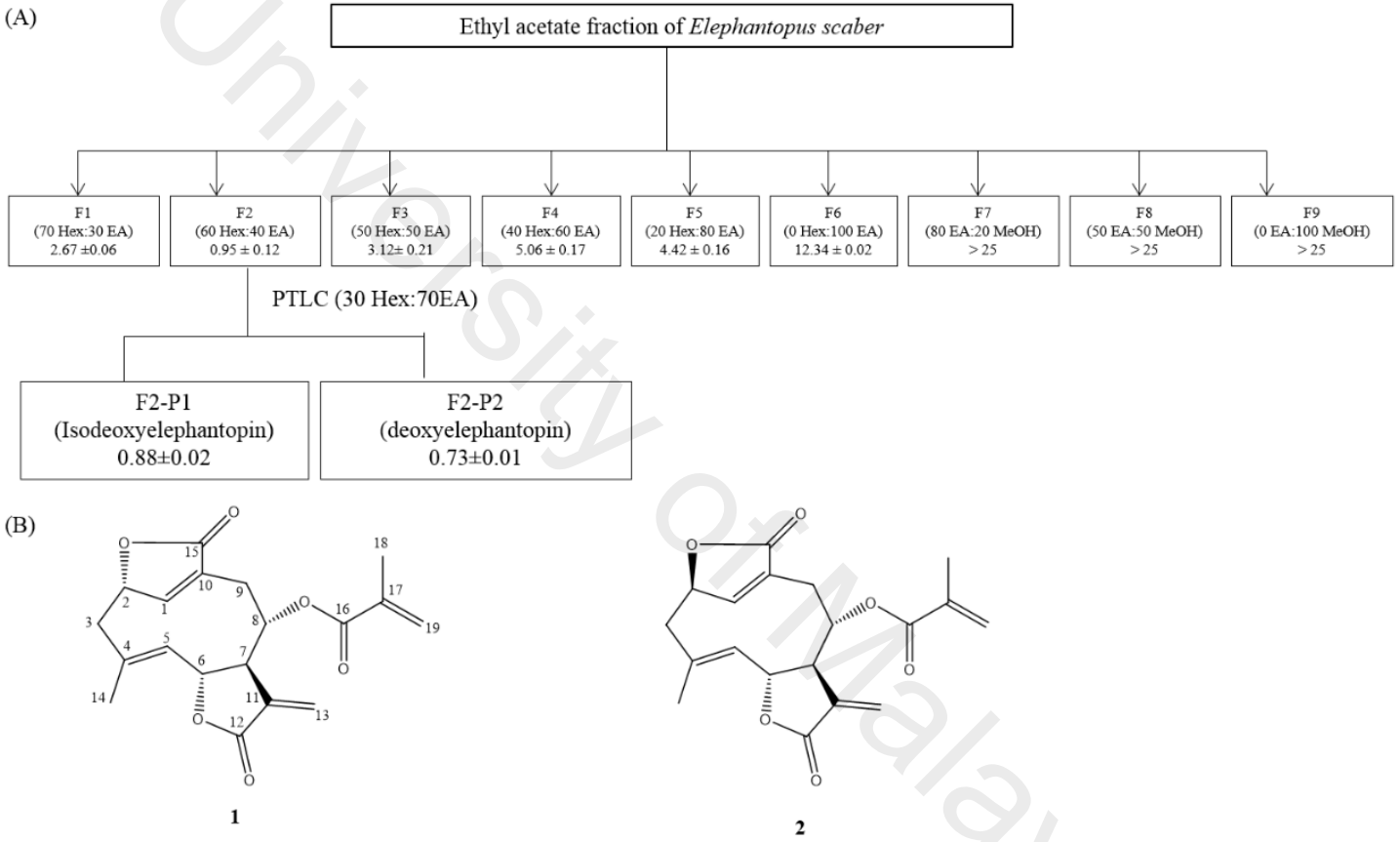


Figure 4.12: Bioactive phytochemical constituents from ethyl acetate fraction of *E. scaber were* isolated and identified. (A) The chart illustrated the detailed isolation of bioactive constituents via bioassay-guided approach. Each fraction was subjected for cytotoxic determination by using MTT assay in HCT116 cells. The IC_{50} values were presented as mean ± S.E. (n=9). Each fractions was resuspended with DMSO and the final concentration of DMSO was maintained below 0.5% v/v throughout all experiments. (B) Chemical structure of isodeoxyelephantopin 1 and

Table 4.3: IsoDET and compound 1 were analyzed and compared by 1H and 13C NMR of (δ in ppm; 400 MHz in $CDCl_3$)

Position	δH, J (Hz) isoDET (But et al, 1997)	δH, J (Hz) compound 1	δC isoDET (But et al, 1997)	δC compound 1
1	7.16, s	7.14, s	150.0	149.4
2	5.38, d (4.5)	5.37, d (4.5)	79.4	79.6
3a	2.39, dd (4.5, 14.0)	2.39, dd (4.5, 14.6)	40.0	40.2
3b	2.94, d (14.0)	2.93, d (14.6)		
4			135.4	135.5
5	5.13, d (10.0)	5.13, d (10.1)	125.2	125.5
6	5.17, d (10.0)	5.17, d (10.1)	78.7	78.8
7	3.15, m	3.15, m	49.8	50.0
8	4.53, ddd (4.0, 4.0, 12.0)	4.52, ddd (4.0, 4.0, 12.4)	74.0	74.1
9a	2.74, dd (4.0, 12.0)	2.74, dd (4.0, 12.4)	30.0	30.2
9b	3.06, dd (12.0, 12.0)	3.04, dd (12.0, 12.4)		
10			131.4	131.6
11			134.0	134.1
12			169.4	169.6
13a	5.65, d (3.2)	5.64, d (3.6)	123.0	123.3
13b	6.20, d (4.0)	6.20, d (4.1)		
14	1.78, s	1.78, s	21.5	21.7
15			174.3	174.4
16			166.5	166.7
17			135.4	135.5
18	1.93, s	1.93, s	18.1	18.3
19a	5.67, s	5.67, s	126.8	127.0
19b	6.15, s	6.14, s		

Table 4.4: DET and compound 2 were analyzed and compared by ^{1}H and ^{13}C NMR of (δ in ppm; 400 MHz in $CDCl_3$)

Position	δ_H, *J* (Hz) DET (Than et al, 2005)	δ_H, *J* (Hz) compound **2**	δ_C DET (Than et al, 2005)	δ_C compound **2**
1	7.08, br *s*	7.06, br *s*	153.5	153.3
2	5.46, *td* (1.8, 3.9)	5.46, *td* (1.8, 4.1)	81.4	81.4
3a	2.69, *ddd* (1.2, 2.1, 13.4)	2.70, *ddd* (1.2, 2.1, 13.4)	41.2	41.5
3b	2.85, *dd* (4.5, 13.8)	2.86, *dd* (4.6, 13.8)		
4			135.5	135.7
5	4.77, br *d* (10.5)	4.78, br *d* (10.5)	133.6	133.9
6	5.13, *dd* (8.1, 10.5)	5.14, *dd* (8.2, 10.5)	78.0	78.0
7	2.94, *dt* (3.6, 7.5)	2.94, *dt* (3.6, 7.7)	52.2	52.5
8	4.65, *ddd* (2.1, 3.6, 11.4)	4.65, *ddd* (1.8, 3.6, 11.6)	71.5	71.6
9a	2.78, *d* (12.3)	2.79, *d* (12.4)	33.4	33.7
9b	3.02, *ddd* (1.7, 3.0, 12.6)	3.01, *ddd* (1.7, 3.0, 12.5)		
10			128.3	128.7
11			134.0	134.1
12			169.3	169.4
13a	5.65, br *d* (3.3)	5.65, br *d* (3.2)	123.6	123.8
13b	6.23, br *d* (3.9)	6.23, br *d* (3.6)		
14	1.85, *d* (1.5)	1.84, *d* (1.4)	20.0	20.2
15			172.5	172.5
16			166.4	166.5
17			135.9	136.1
18	1.93, *dd* (1.2, 1.4)	1.93, *s*	18.2	18.3
19a	5.66, *d* (1.5)	5.66, *d* (1.8)	126.6	126.8
19b	6.14, *t* (1.2)	6.14, *t* (1.2)		

Table 4.5: IC_{50} values of deoxyelephantopin, isodeoxyelephantopin and 5-fluorouracil in HCT116 cells and CCD841-CoN normal cells.

Cell lines	IC_{50} (µg/mL)		
	Deoxyelephantopin	Iso-deoxyelephantopin	5-fluorouracil[a]
HCT116	0.73 ±0.01	0.88 ± 0.02	0.73 ± 0.02
CCD841-CoN	21.69 ± 0.92	NA	> 25

The data represented as mean ± S.E. of three independent experiments (n = 9).

ND: Not determined.

[a] 5-fluorouracil served as positive control.

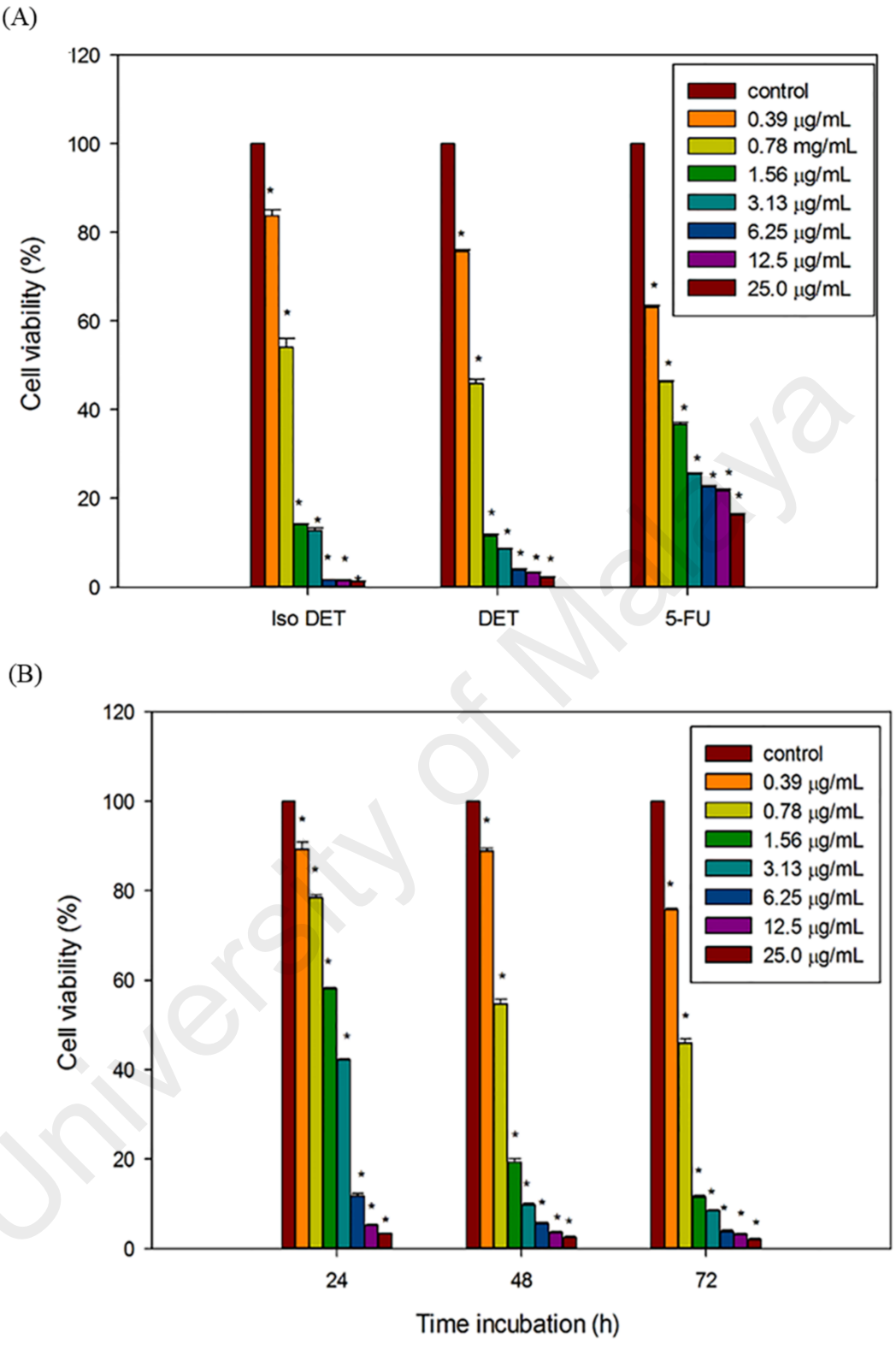


Figure 4.13: The cytotoxic effect of deoxyelephantopin and isodeoxyelephantopin in HCT116 cells. (A) The bar chart depicted the percentage of cell viability upon exposure to varying concentrations of DET, isoDET and 5-FU (0.39- 25.0 µg/mL) in HCT116 cells for 72 h. (B) The bar chart displayed the time course cell viability when treated with DET against HCT116 cells at 24, 48 and 72 h. The data represented as mean ± S.E. of three independent experiments (n = 9). Asterisks denote as significantly different value when compared to control (* $p < 0.05$).

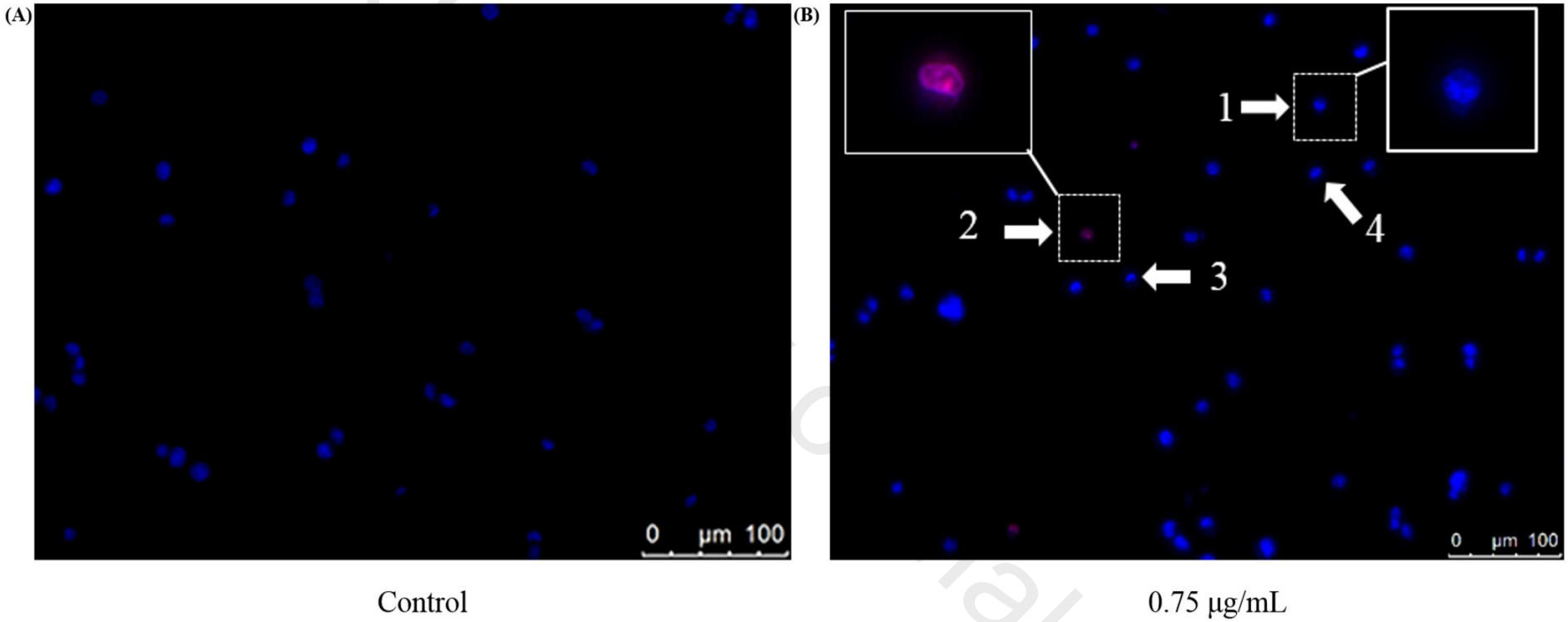


Figure 4.14: The nuclear morphological changes by deoxyelephantopin in HCT116 cells. (A) HCT116 cells were treated with DMSO as vehicle control (B) HCT116 cells were treated with 0.75 µg/mL of DET for 24 h.
Magnification: 200X Arrow 1 chromatin condensation, 2 late apoptosis, 3 cell shrinkage, 4 DNA fragmentation, 5 necrosis

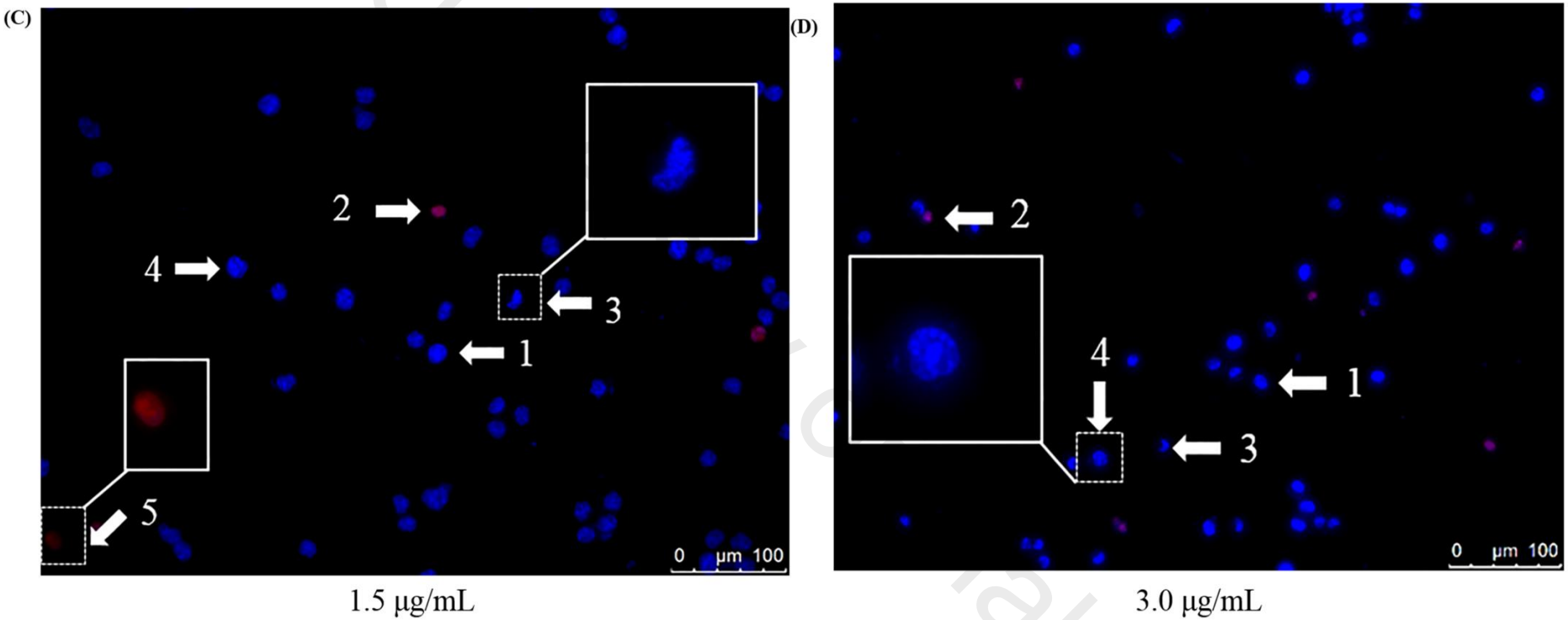


Figure 4.14, continued. (C) HCT116 cells were treated with 1.5 µg/mL of DET and (D) 3.0 µg/mL of DET followed by Hoechst 33342 and PI staining and detection under fluorescence microscope.
Magnification: 200X Arrow 1 chromatin condensation, 2 late apoptosis, 3 cell shrinkage, 4 DNA fragmentation, 5 necrosis

one of the biochemical hallmarks in apoptosis which can be detected by Annexin V-FITC/PI staining. Flow cytometric plots were divided into four different phases in which lower left is viable ($Annexin^-/PI^-$), lower right is early apoptosis ($Annexin^+/PI^-$), upper right is late apoptosis ($Annexin^+/PI^+$) and upper left is necrosis ($Annexin^-/PI^+$). As displayed in flow cytometric results, most of the cells were accumulated in the lower left quadrant indicating healthy and viable cells for the untreated control group (Figure 4.15(a)). However, DET treatment resulted in the change of phospholipid membrane symmetry evidenced by the shift from viable to early and late apoptosis. A substantial accumulation of Annexin V positive stained cells which consist of early and late apoptotic cells along with a dose-dependent decrease of viable cells were observed upon treatment with DET (Figure 4.15(b)). Notably, DET markedly elevated to 63.63 ± 2.03 % at highest concentration of DET (3.0 µg/mL). These findings suggested that DET induced externalization of phosphatidylserine in HCT116 cells.

4.16 Activation of apoptosis-related proteins by DET

Proteolytic cleavage of PARP is induced by the activation of caspase-3 resulting in cell death via apoptosis. To elucidate the apoptosis underlying mechanism conferred by DET in HCT116 cells, cleaved caspase-3 and cleaved PARP which are characterized as apoptosis determinants were investigated by using western blot analysis. In line with the apoptosis induction, caspase-3 and PARP cleavage were remarkably increased in response to DET (Figure 4.16 (a)). Significant accumulation of cleaved caspase-3 and cleaved PARP protein by 2.07 ± 0.11 and 3.59 ± 0.16 folds, respectively, were found at the highest concentration of DET, 3.0 µg/mL in HCT116 cells when compared to untreated control (Figure 4.16 (b) & (c)). Therefore, collective findings indicated that DET which resulted in a decline in cell viability of HCT116 cells was attributed to the execution of apoptotic cell death.

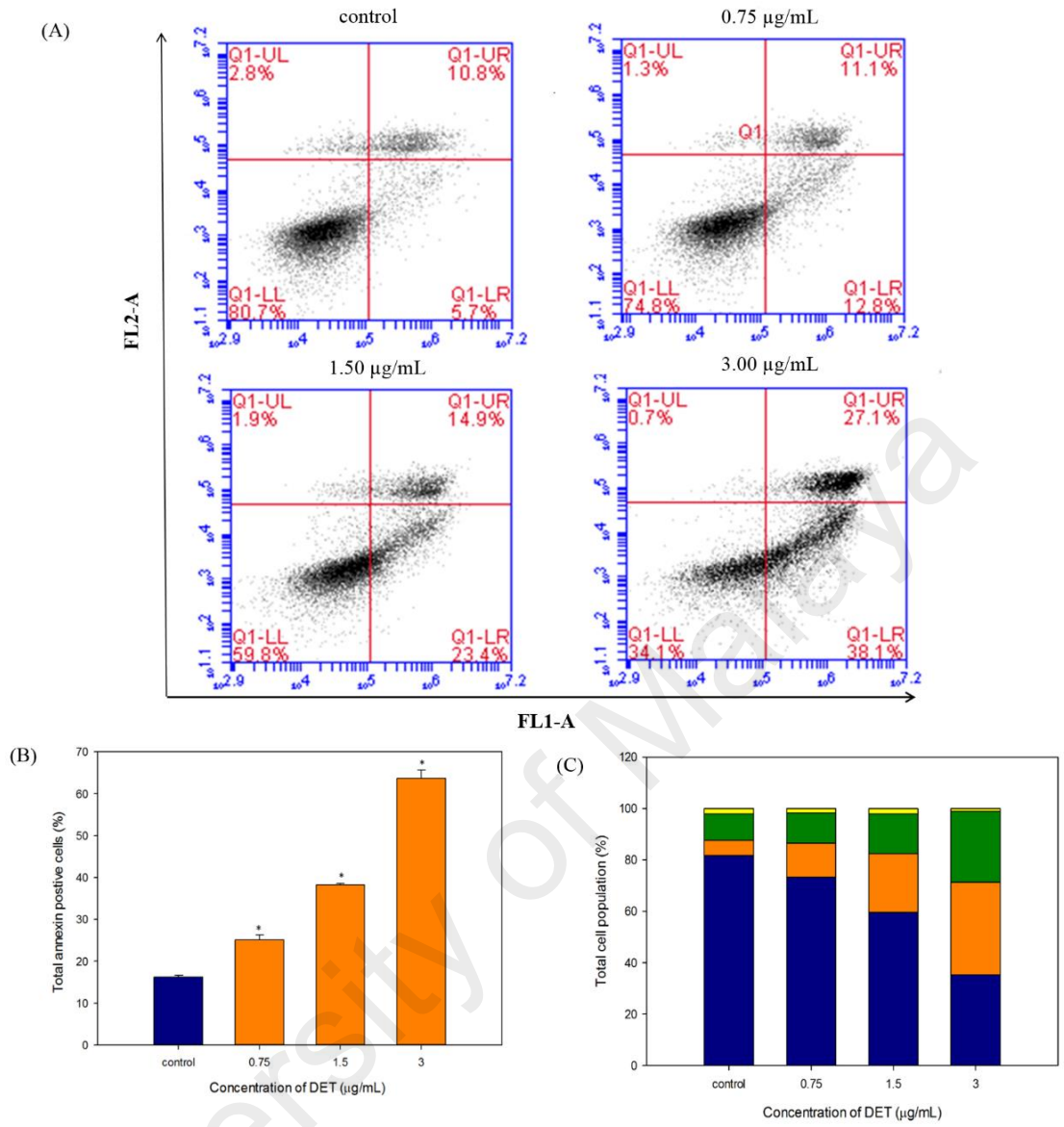


Figure 4.15: Externalization of phosphatidylserine induced by DET (A) The flow cytometric dot plot demonstrated the distribution of viable, early apoptotic, late apoptotic and necrotic cells in increasing concentrations of DET via Annexin V-FITC/PI staining at 24 h. (B) The bar chart displayed the percentage of Annexin V positive cells. (C) The bar chart depicted the proportion of viable, early apoptotic, late apoptotic and necrotic cells. The data represented as mean ± S.E. from three individual experiments. Asterisks denote as significantly different value when compared to control (*$p<0.001$).

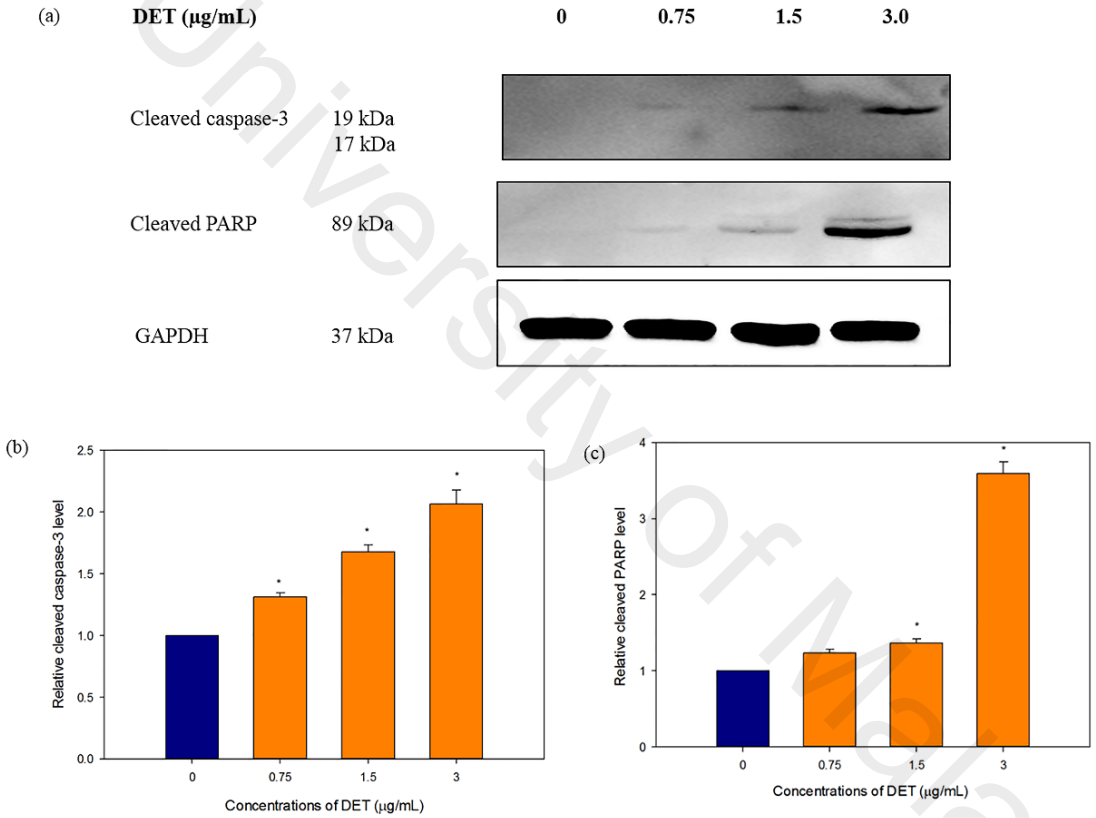


Figure 4.16: Modulatory effect of deoxyelephantopin on the expression of apoptosis-related proteins in HCT116 cells. (A) Representative western blot band intensity of cleaved caspase-3 and cleaved PARP proteins. The bar chart illustrated the relative expression of (B) cleaved caspase-3 and (C) cleaved PARP. The data represented as mean ± S.E. from three individual experiments. Asterisks denote as significantly different value when compared to control (*$p<0.001$).

4.17 Effect of DET on the induction of cell cycle arrest

In order to verify the causative factor related to anti-proliferative effect by DET, the distribution of cell cycle was investigated by flow cytometry. As illustrated in Figure 4.6, the data revealed that DET arrested cell cycle progression at both S and G2/M phases in HCT116 cells. A significant concentration-dependent accumulation of cells in the S phase from 23.21 ± 0.38 to 28.17 ± 0.23 and 29.25 ± 0.21 %, was resulted upon exposure to 1.5 and 3.0 µg/mL of DET, respectively at 24 h (Figure 4.17). In addition, cell population at G2/M was also found significantly augmented to 23.27 ± 0.25 and 30.7 ± 0.21 % at 1.5 and 3.0 µg/mL of DET, respectively when compared to untreated cells (20.89 ± 0.33 %). Concurrently, an increment of hypodiploidal cell population (sub G1/apoptosis) accompanying a reduction in G1 phase was noted when treated with DET. In light of the prominent sub G1 apoptotic peak, DET induced apoptosis at the same time which is corroborated by the corresponding externalization of phosphatidylserine, activation of caspase-3 and cleavage of PARP. Accordingly, results implied that DET blocked the cell cycle progression at S and G2/M phases leading to the inhibition on HCT116 cell proliferation accompanied by an increase in subG1 phase indicating that suppression on cellular mitosis induced the initiation of apoptosis.

4.18 Modulation of the protein expression associated with cell cycle by DET

Next, to decipher the blockade on cell cycle progression in HCT116 cells, cell cycle-related protein expression was assessed by western blot analysis. Our results revealed that CDK4, CDK2 and cyclin A2, B1, E2 and D1 which are essential mediators in governing the progression of cell cycle were found markedly downregulated in DET-treated cells (Figure 4.18(a)). The transition of cell cycle is attenuated by DET-induced reduction on cyclins and CDKs protein expression in HCT116 cells (Figure 4.18(b)). Conversely, treatment with DET did not significantly alter the protein expression of CDK6 as shown

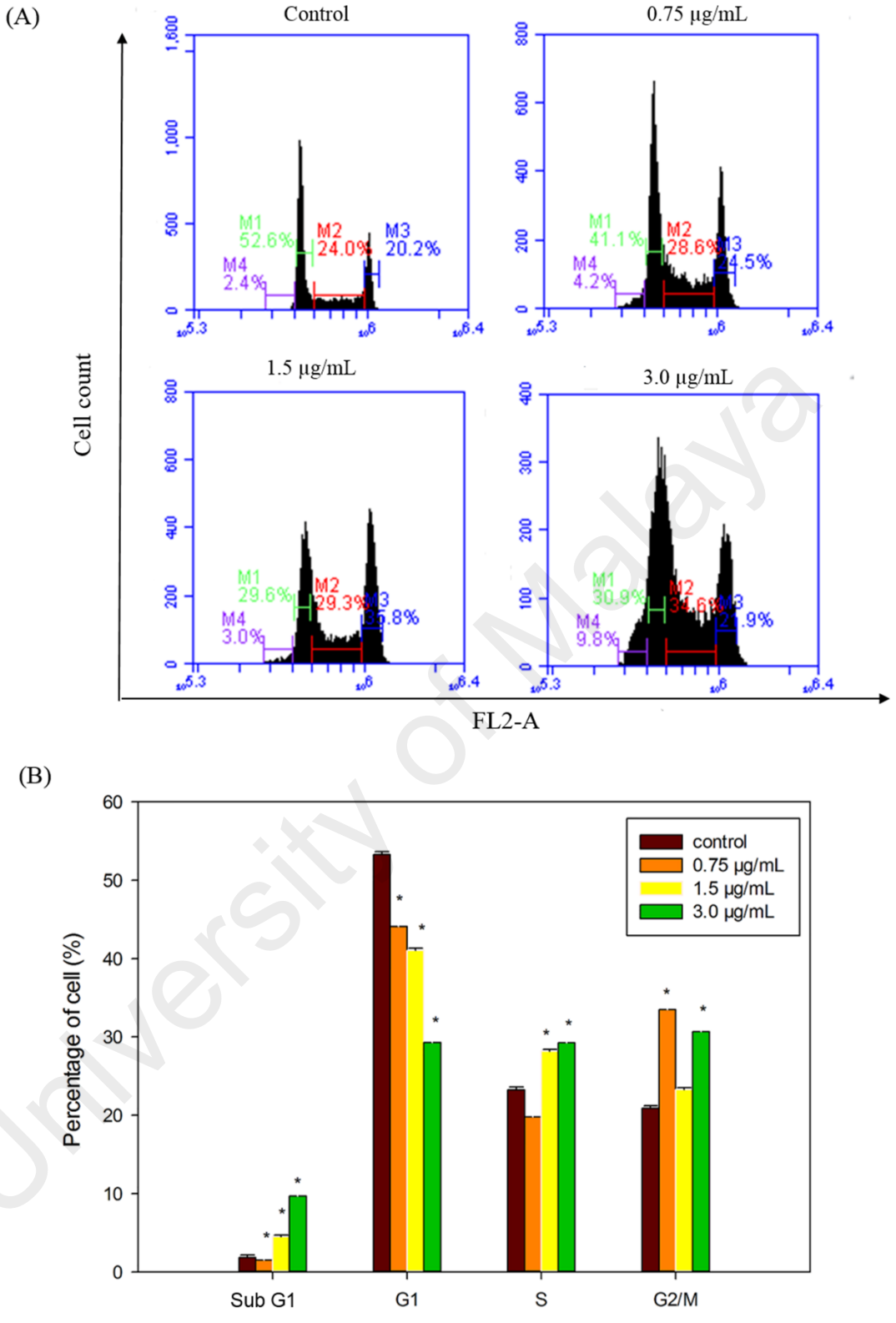


Figure 4.17: The cell cycle distribution upon exposure of DET in HCT116 cells. HCT116 cells were exposed to 0.75, 1.5 and 3.0 µg/mL of DET for 24 h. The data represented as mean ± S.E. from three different experiments. Asterisks denote as significantly different value when compared to control (*p<0.001).

in Figure 4.18(b). Additionally, cell cycle inhibitors p21 and phospho-p53, were dose-dependently elevated upon incubation of DET in HCT116 cells (Figure 4.18(b)) which further supported by the flow cytometry data.

4.19 DET mediated apoptosis via intrinsic and extrinsic pathways

Intrinsic and extrinsic pathways are two focal apoptotic pathways which involve mitochondrial and death receptor, respectively. In a previous study, findings suggested that DET possessed anti-proliferative effect via inducing cell cycle arrest and apoptosis in HCT116 cells. Thus, to elucidate the underlying apoptosis mechanism, apoptosis-related protein expression was investigated by using protein array and western blot analysis. Protein array data demonstrated that DET upregulated p53, p21, caspase-3, Fas, DR4, DR5 and IGFBP-5 along with the downregulation of XIAP, survivin and IGF-1sR in a dose-dependent manner (Figure 4.19(a) & (b)).

To further substantiate and precisely decipher the underlying mechanisms, apoptosis-related protein expression was evaluated by western blot analysis. Western blot results showed that cleaved caspase-3, DR5 and Fas protein expression was significantly upregulated in a time-dependent manner whereas procaspase-9 and -10 was time-dependently decreased which were corroborated with the protein array results. Consequently, as depicted in Figure 4.19, inhibitor of apoptosis proteins (IAPs) such as XIAP and survivin were found downregulated in response to DET treatment in HCT116 cells which is in accordance with the protein array findings (Figure 4.19(c)).

The Bcl-2 family proapoptotic proteins (e.g. Bax and Bak) and antiapoptotic proteins (e.g. Bcl-2 and Bcl-xL) are crucial in governing apoptosis. Hence, the effect of DET on the protein expression of Bcl-2 family proteins was investigated. It was found that DET caused Bak and Bax protein expression to increase significantly whereas Bcl-2 and Bcl-

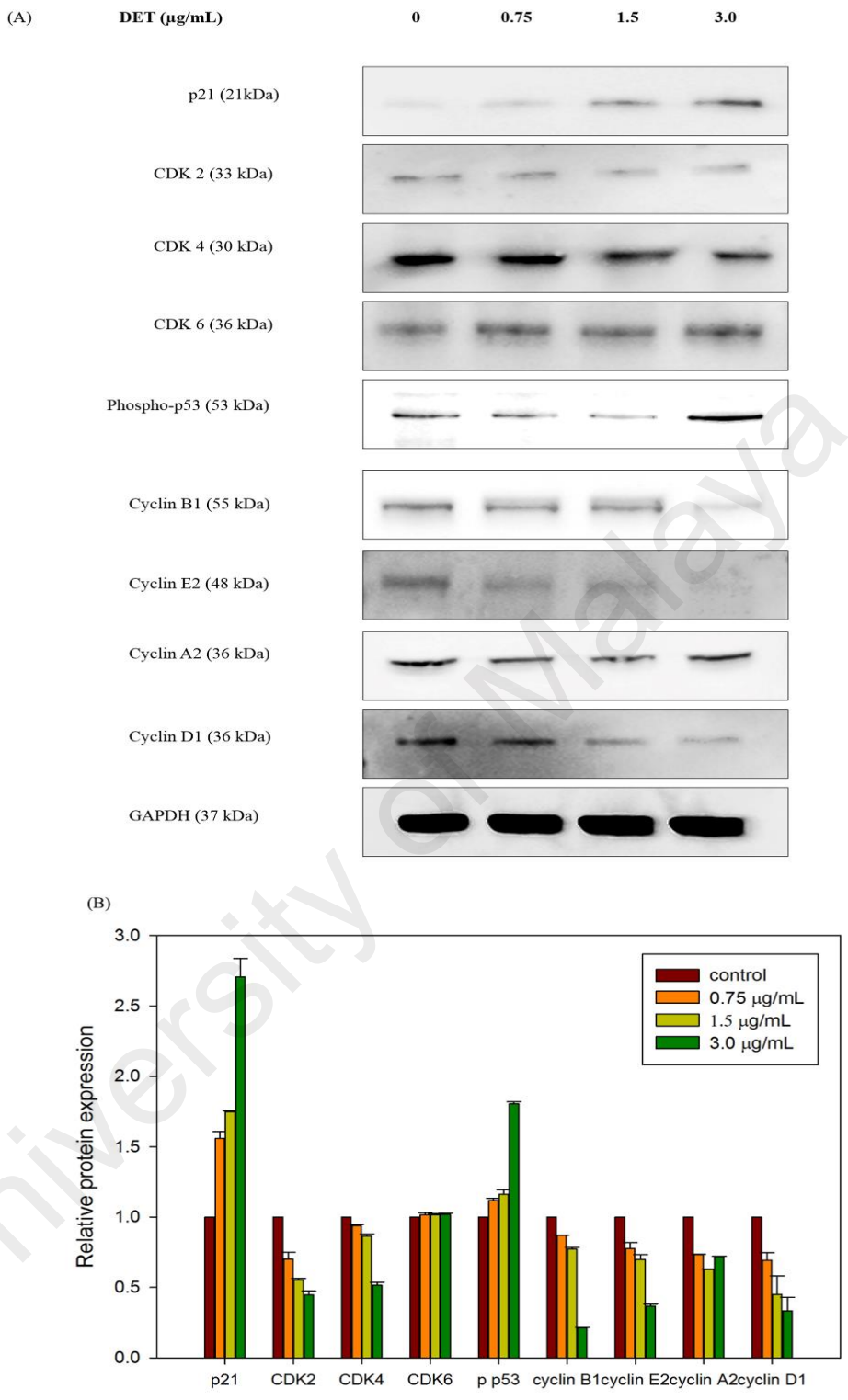


Figure 4.18: The modulatory effect on the cell cycle-related protein expression in HCT116 cells. Exposure of 0.75, 1.5 and 3.0 µg/mL of DET in HCT116 cells for 24 h. (A) Representative western blot images of p21, phospho-p53, CDK2, CDK4, CDK6, cyclin B1, cyclin E2, Cyclin A2 and Cyclin D1 protein expression. (B) The bar chart depicted the respective protein expression levels. The data represented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value when compared to control (*p<0.001).

xL protein expression was evidently decreased at 24 h (Figure 4.19(c)). Dysregulation of Bcl-2 family members will lead to the depolarization of mitochondrial membrane potential, an early apoptotic hallmark, which can be detected by MITO-ID fluorescent dye. As depicted in Figure 4.19(d), the untreated cells displayed the most abundant orange aggregates in mitochondria. However, in the DET-treated cells, increasing intensity of green monomer fluorescence in the cytoplasm that was accompanied by the loss of orange aggregate fluorescence was observed from the beginning at the lowest concentration of DET (0.75 µg/mL). The percentage of orange aggregates almost completely decreased to 9.65 ± 1.26 % when exposed to the highest concentration of DET treatment (3.0 µg/mL) as compared to the control untreated cells (81.04 ± 4.39 %). Accordingly, DET caused a dose-dependent loss of mitochondrial membrane potential suggesting the association of mitochondria-mediated apoptosis in HCT116 cells. Collectively, these findings demonstrated that DET mediated intrinsic and extrinsic pathways leading to apoptosis.

4.20 Oxidative stress

4.20.1 The generation of reactive oxygen species level and its involvement in apoptosis

Reactive oxygen species, a mediator which is crucial in several cell death modalities including apoptosis, autophagy and necrosis. Thus, the ROS level was quantified by using DCFH-DA dye upon exposure of DET flow cytometry. The increase in intracellular ROS was represented by the right shift of the histogram. The level of intracellular ROS was found potently elevated by 1.73 ± 0.04, 3.72 ± 0.15 and 4.66 ± 0.16 folds, at 0.75, 1.5 and 3.0 µg/mL of DET in HCT116 cells (Figure 4.20 (a) & (b)). To further confirm that the correlation between apoptosis and ROS, NAC inhibitor was used

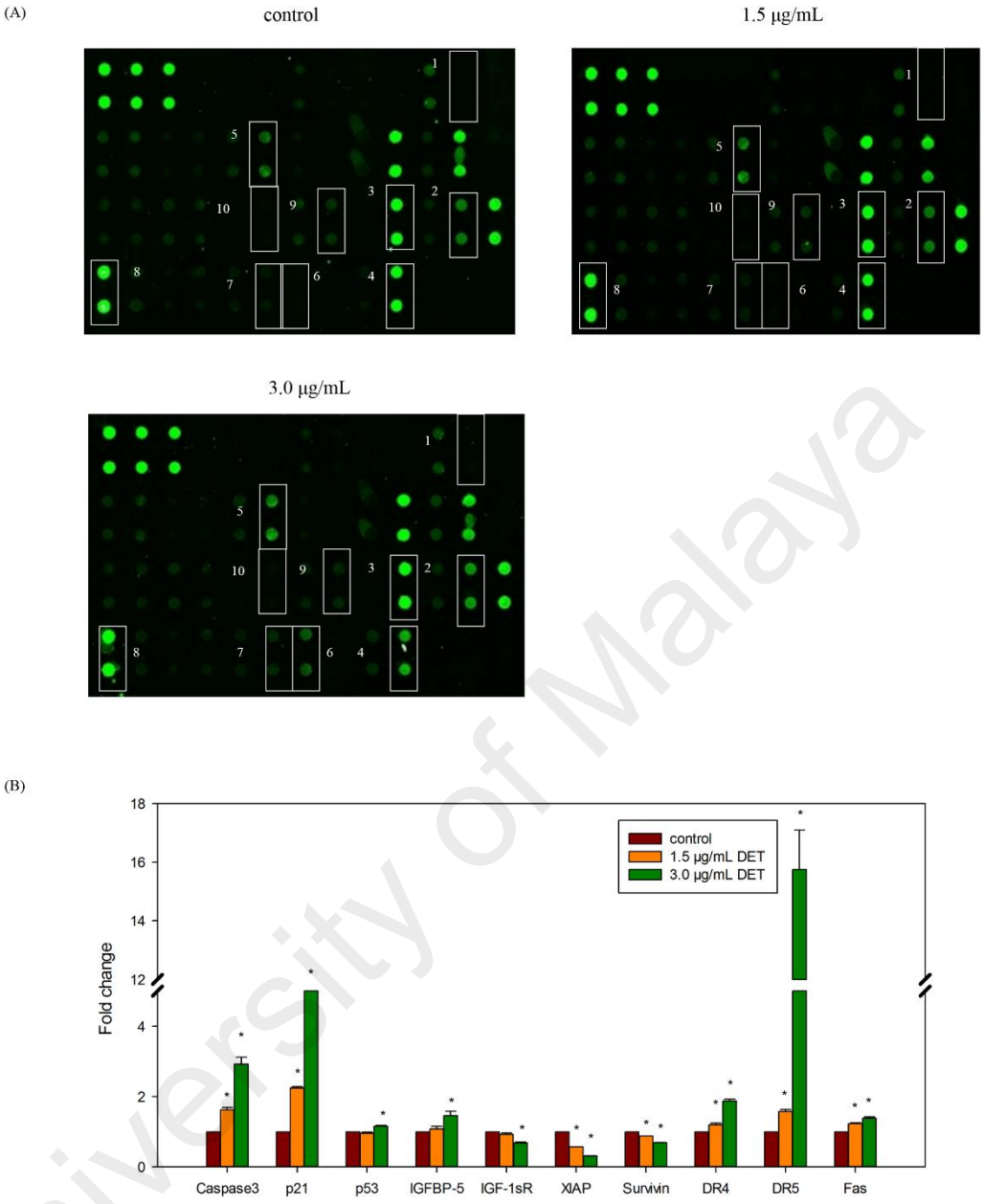


Figure 4.19: Induction of apoptosis by DET via intrinsic and extrinsic pathway. (A) Protein array representatives' images of the fluorescence intensity on the apoptosis-related protein expression after treatment with DET. (B) The bar chart depicted the fold change of apoptosis-related proteins. 1. Caspase-3; 2. p53; 3. p21; 4. XIAP; 5. Fas; 6. TRAILR-1; 7.TRAILR-2; 8. survivin; 9. IGF-1sR; 10. IGFBP-5

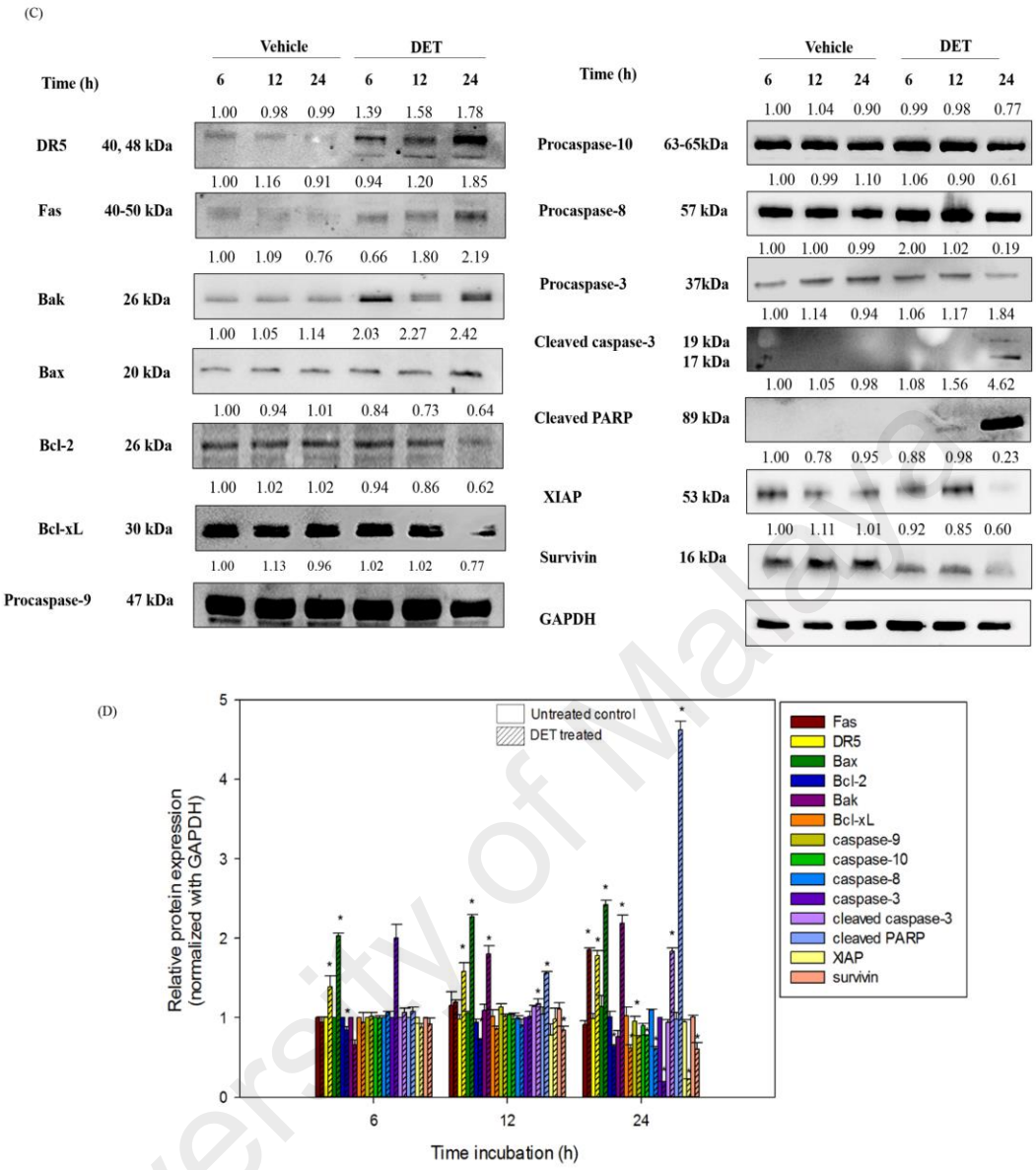


Figure 4.19, continued. (C) Representative band intensity of respective proteins including DR5, Fas, Bak, Bax, Bcl-2, Bcl-xL, cleaved caspase-3, caspase-3, caspase-8, caspase-9, caspase-10, XIAP and survivin. GAPDH as internal control. (D) Bar chart showed relative apoptosis-related protein expression.

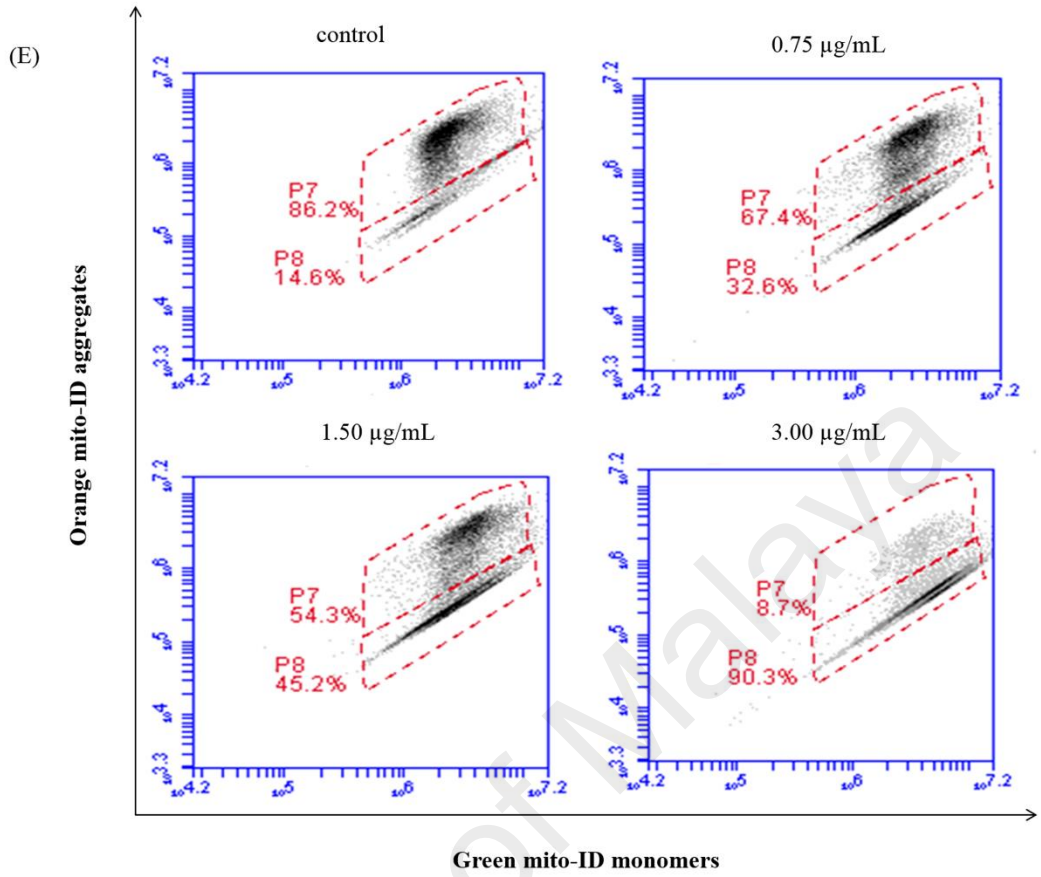


Figure 4.19, continued. (E) Flow cytometric dot plot indicated the effect of DET on mitochondrial membrane potential. The data presented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value as compared to control (*p< 0.05).

prior to DET treatment. Results demonstrated that the change in ROS was abrogated in presence of NAC and DET (Figure 4.20(c)). Apart from this, the effect of DET on viability of HCT116 cells with and without NAC were assessed at 24 h. Based on the results, DET-induced reduction of HCT116 cells was found to be reversed in the presence of NAC (Figure 4.20(d)). Besides, in the presence of NAC, the apoptotic protein expression including cleaved caspase-3, cleaved PARP and DR5 were abrogated whereas the procaspase-9 and procaspase-10 was elevated when HCT116 cells were exposed to DET (Figure 4.20(g) & (h).

4.20.2 The activation of ROS-dependent MAPK pathway in DET-induced apoptosis

MAPK family members including p38, ERK1/2 and JNK play key roles in the regulation of apoptotic cell death and cell proliferation. The effect of DET on the MAPK pathway in HCT116 cells was evaluated by using western blot analysis. According to Figure 4.20(e) & (f), treatment with DET significantly upregulated p-ERK1/2, p-JNK and p-p38 in a time-dependent manner in HCT116 cells. Growing reports revealed that the potential activation of MAPK pathway may be attributed to the stimulation by ROS. Thus, the association of ROS and DET-induced MAPKs pathway was investigated in this study. Upon pre-treatment with NAC, DET-induced phosphorylation of JNK, p38 and ERK1/2 proteins were attenuated in HCT116 cells (Figure 4.20(g)&(h)). To evaluate the correlation between MAPK pathway and apoptosis, specific inhibitors for p38 (SB202190), JNK (SP600125) and ERK (UO126) were pre-exposed in DET-treated HCT116 cells. Figure 4.20(i) and (j) illustrated that respective inhibitors effectively reversed DET-induced activation of cleaved caspase-3 and cleaved PARP in HCT116 cells. These collective findings suggested that stimulation of ROS is essential for the DET induced activation of MAPK and apoptosis in HCT116 cells.

4.21 Attenuation of PI3K/Akt/mTOR signalling pathway by DET

Targeting survival pathway such as PI3K/Akt/mTOR is found to favor apoptotic cell death mechanism. Hence, the participation of PI3K/Akt/mTOR in DET-induced apoptosis in HCT116 cells was explored. DET demonstrated a time-dependent attenuation of Akt phosphorylation at Ser 473 and Thr 308 as compared to the constant expression of the total Akt level. Subsequently, the effect of DET on the phosphorylation of PTEN and PDK-1 was examined. As shown in Figure 4.21, DET significantly reduced the phosphorylated PTEN (inactivated form of PTEN) and PDK1 protein expression in a time-dependent manner. These findings suggested DET activated PTEN along with the inactivated PDK1. Subsequently, mTOR a downstream mediator of Akt as well the regulatory subunits raptor and rictor were evaluated upon treatment with DET. DET was found to decrease the phosphorylation of mTOR in HCT116 cells. Simultaneously, both the rictor and raptor expression was reduced significantly at 12 and 24 h when HCT116 cells were treated with 3.0 μg/mL of DET. These findings indicated that DET mitigated PI3K/Akt/mTOR signalling pathway to promote apoptosis in HCT116 cells.

4.22 DET mediated autophagy in HCT116 cells

Autophagy is one of the focus as a promising strategy in developing anticancer drugs. Accordingly, AO staining, TEM analysis and the autophagy related protein expressions (LC3 A/B, Atg7, Atg5 and p62) detected by western blot analysis were carried out to verify the involvement of autophagy in DET-treated cells. As illustrated in Figure 4.22(a), a substantial number of bright red acidic vesicles resembling autolysosomes was detected in HCT116 cells upon treatment with DET. In addition, cytological evidences as evaluated by TEM revealed the occurrence of double-membrane autophagosomes containing residual digested material and empty vacuoles in response to DET in HCT116

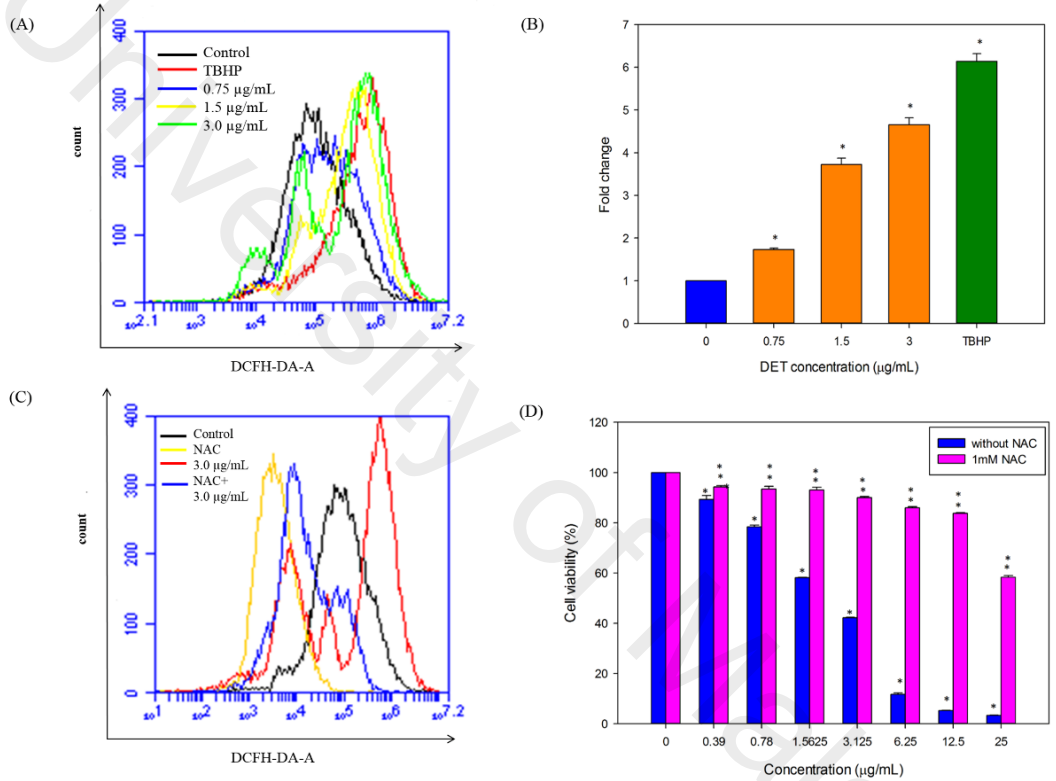
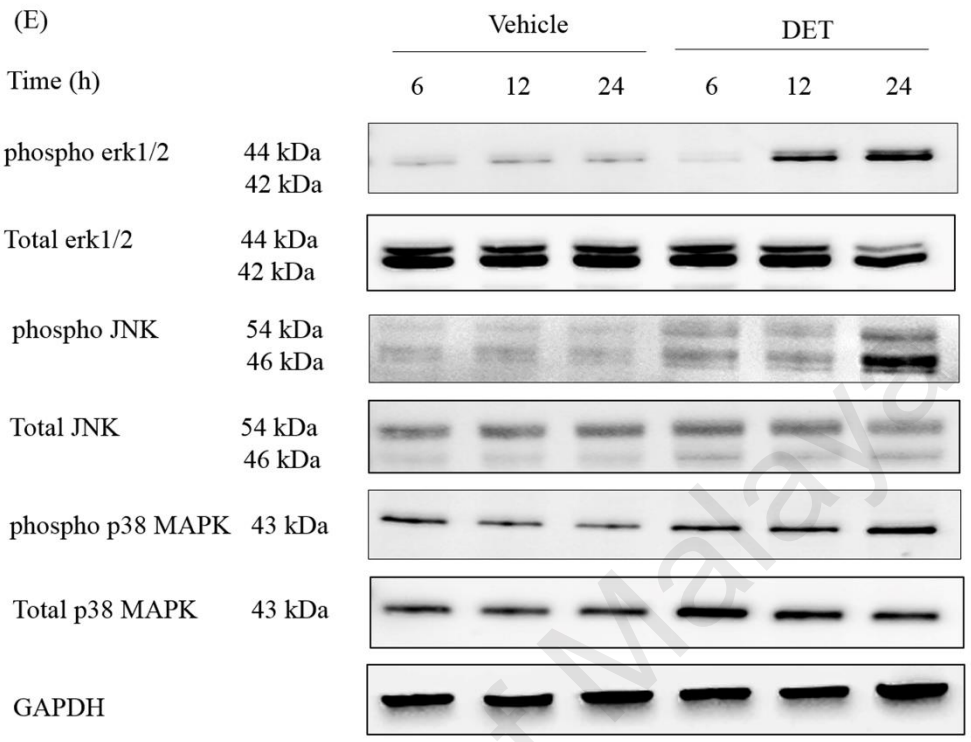


Figure 4.20: ROS-dependent MAPK and apoptotic pathways triggered by DET in HCT116 cells. (A) Histogram demonstrated the fluorescence intensity of DCFH-DA indicating intracellular ROS level upon exposure with different concentrations of DET (0.75, 1.5 and 3.0 µg/mL). (B) The bar chart depicted the effect of DET on the intracellular ROS fold change. (C) The histogram displayed the presence or absence of 1 mM NAC on DET-treated cells. (D) The bar chart represented the effect of DET (0.39 – 25 µg/mL) on the cell viability of HCT116 cells in presence or absence of 1mM NAC by using MTT assay.



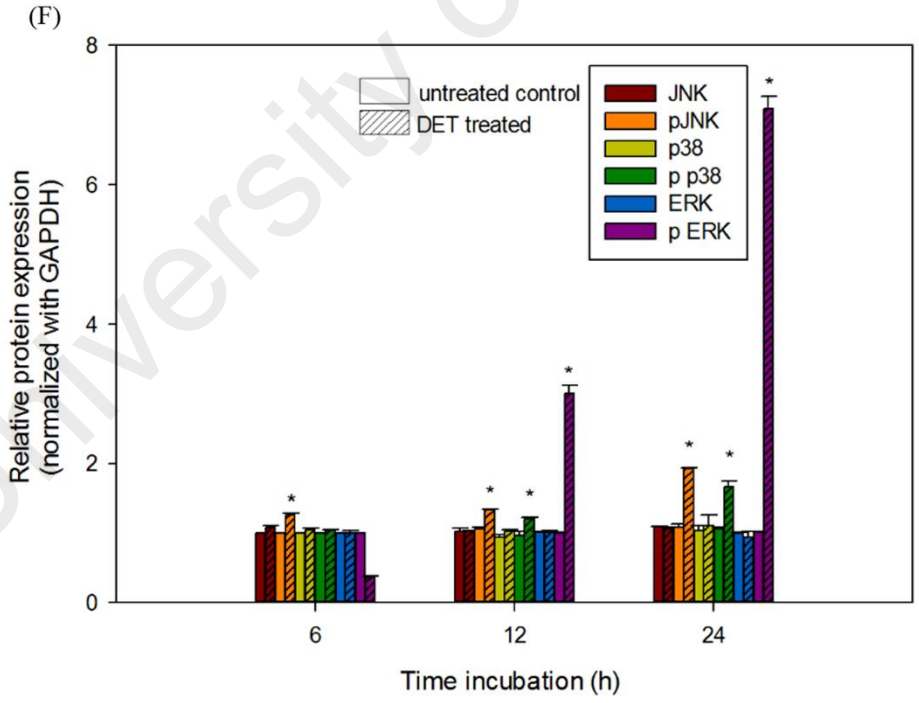


Figure 4.20, continued. (E) The expression of phospho ERK1/2, phospho JNK, phospho p38, ERK1/2, JNK and p38 were assessed by western blot analysis after DET treatment in HCT116 cells. (F) Bar chart showed the relative expression of MAPKs proteins.

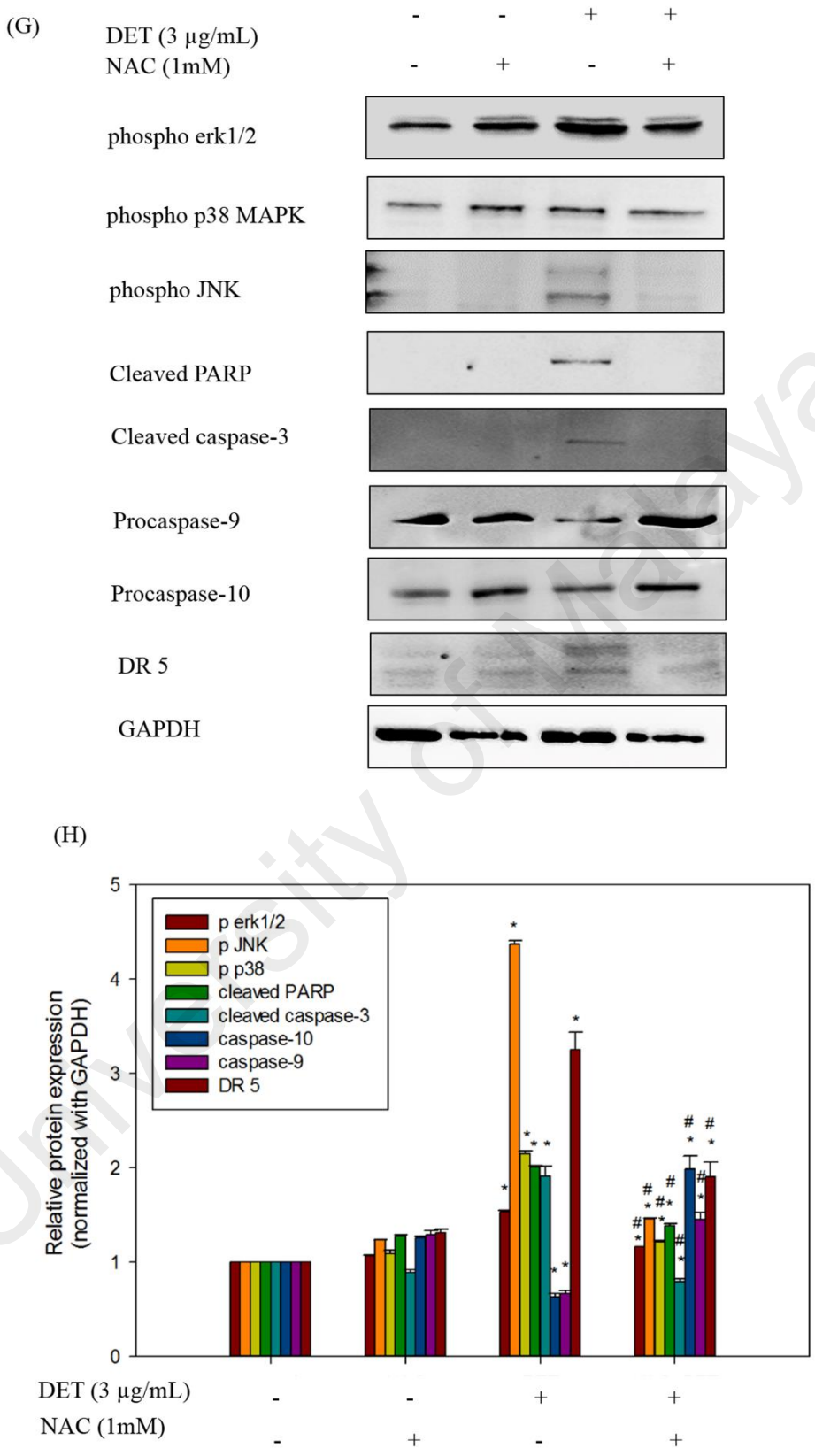


Figure 4.20, continued. (G) The expression of phospho ERK1/2, phospho JNK, phospho p38, cleaved PARP, cleaved caspase-3, procaspase-9, procaspase-10 and DR5 with or without pre-treatment of NAC were assessed by western blot analysis. (H) Bar chart indicated respective protein expression upon pre-incubation of NAC.

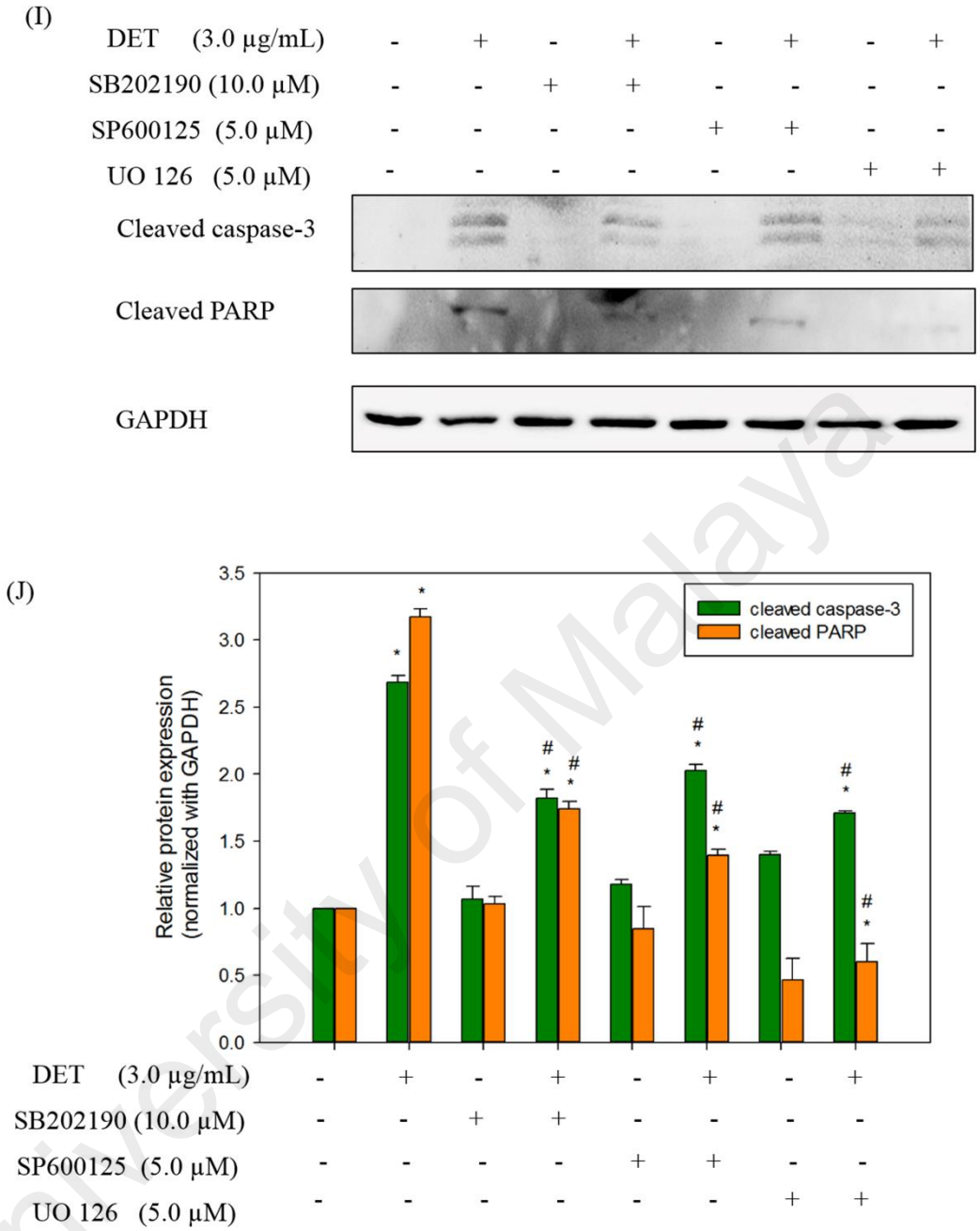


Figure 4.20, continued. (I) The expression of cleaved caspase-3 and cleaved PARP with or without pre-treatment of SB202190, SP600125 and UO126 specific inhibitors, respectively were assessed by western blot analysis. (J) Bar chart showed relative apoptosis-related protein in presence of MAPKs inhibitors. GAPDH was used as internal control. The data presented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value as compared to control (**p*< 0.05). Hashtags denote significantly different compared to DET treatment (#*p*<0.05).

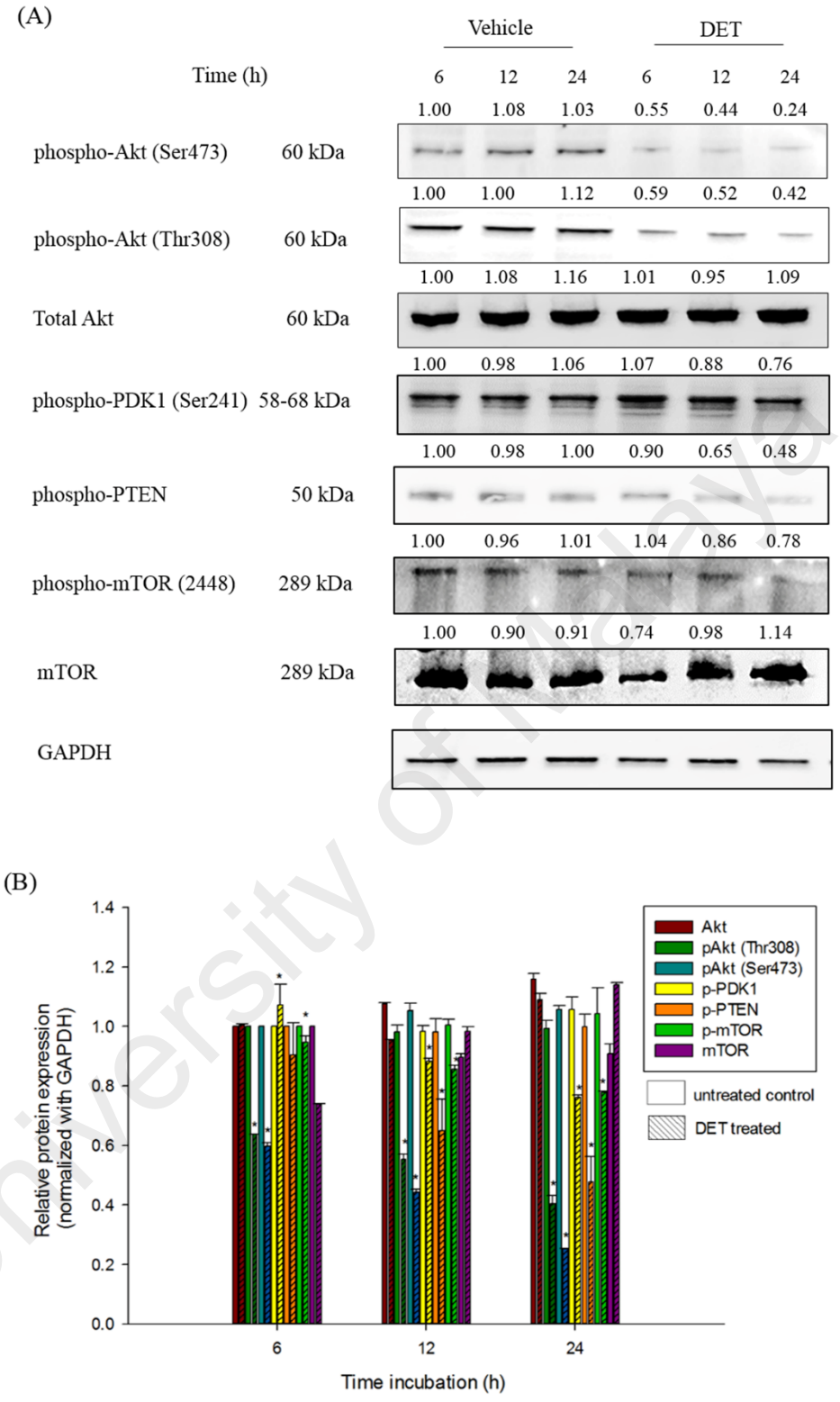


Figure 4.21: The effect of DET on the mitigation of PI3K/Akt/mTOR signalling pathway. DET was exposed to HCT116 cells at 6, 12 and 24 h. (A) Representative band intensity of respective proteins including Akt, p-Akt (Ser473), p-Akt (Thr308), p-PDK1, p-PTEN, p-mTOR. (B) The bar chart represented the relative protein expression of respective proteins. GAPDH was used as internal control. The data presented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value as compared to control (*p< 0.05).

cells (Figure 4.22(b)). To further substantiate the event of autophagy, autophagy-related proteins were assessed. The present findings demonstrated that DET markedly induced accumulation of LC3A/B II, Atg7 and Atg5 (Figure 4.22(c)). In contrast, a decline in autophagic adaptor p62 protein expression was observed when HCT116 cells were treated with DET. Subsequently, the understanding on the role of ROS oxidative stress in autophagy was examined in the presence of NAC in DET treated cells. Based on the TEM evidences, DET-induced accumulation of autophagosomes was abrogated upon stimulation of ROS scavenger, NAC. This was further substantiated by the AO-stained cells in which the red acidic vesicles induced by DET was not detected. In addition, NAC potently inhibited the induction of autophagy as evidenced by DET-induced accumulation of LC3A/B II was reversed and conversely, p62 protein expression was restored by NAC in HCT116 cells (Figure 4.22(d)). These present findings suggested that induction of autophagy by DET was triggered in response to the oxidative stress, ROS in HCT116 cells.

4.23 The interplay between autophagy and apoptosis

Growing studies have been demonstrated the crosstalk between apoptosis and autophagy mediates cell death in various cancers. Thus, to explore the association between apoptosis and autophagy, apoptosis inhibitor, z-VAD-FMK, and autophagy inhibitor, 3-MA, were used to evaluate the apoptosis and autophagy-related proteins. Based on the figure 4.22(e), induction of autophagy was effectively inhibited by 3-MA which was evidenced by the decrease in lipidation of LC3 A/B II. On the other hand, when autophagy is attenuated, the occurrence of apoptosis persists to a certain extent in DET-treated cells whereby 3-MA moderately reduced the cleavage of caspase-3 and PARP. Conversely, DET-induced apoptosis was significantly suppressed by apoptosis inhibitor, Z-VAD-FMK, supported by the absence of cleaved caspase-3 and cleaved PARP in HCT116 cells (Figure 4.22(f)).

However, DET-induced accumulation of LC3A/B II was further stimulated upon pre-incubation with Z-VAD-FMK. Thus, current findings substantiated the notion that autophagy partially contributes to induction of apoptosis and the mitigation of apoptosis stimulates the induction of autophagy.

4.24 *In vivo* growth inhibitory effect of DET in colorectal carcinoma HCT116 cells

The *in vivo* anti-tumour effect of DET was assessed by colorectal carcinoma xenograft mice. Throughout the experiments, tumour volumes and body weights for control and DET-treated groups were measured. As illustrated in figure 4.23(a), the body weights for all groups of mice did not demonstrate any drastic alterations except for the 50 mg/kg 5-fluorouracil treatment group. Particularly for 5-fluorouracil treatment group, significant reduction in tumour size was observed, however this group of mice died at day 10 suggesting the escalating side effect of 5-fluorouracil (Figure 4.23(b)). In contrast, the administration of 1.25 and 2.5 mg/kg DET potently reduced the size of tumours. The tumour volume was found effectively reduced by 73.48% in response to 2.5 mg/kg DET for a period of 28 days (Figure 4.23(b)). To further explore the *in vivo* model underlying mechanism conferred by DET and verify the *in vitro* findings, IHC staining was carried out. Upon administration of DET, several key markers of apoptosis and autophagy including cleaved caspase-3, LC3 A/B and Bax protein expression were found markedly upregulated. Conversely, IHC evidences revealed the radically decline in proliferating cell nuclear antigen (PCNA)-positive cells as compared to the control group, indicating that DET decreased PCNA protein expression. Moreover, DET significantly downregulated Bcl-2 protein in a dose-dependent manner. Therefore, collective *in vivo* findings were found in line with the *in vitro* findings demonstrating the potential of DET in attenuating the colorectal cancer cell growth via apoptosis and autophagy.

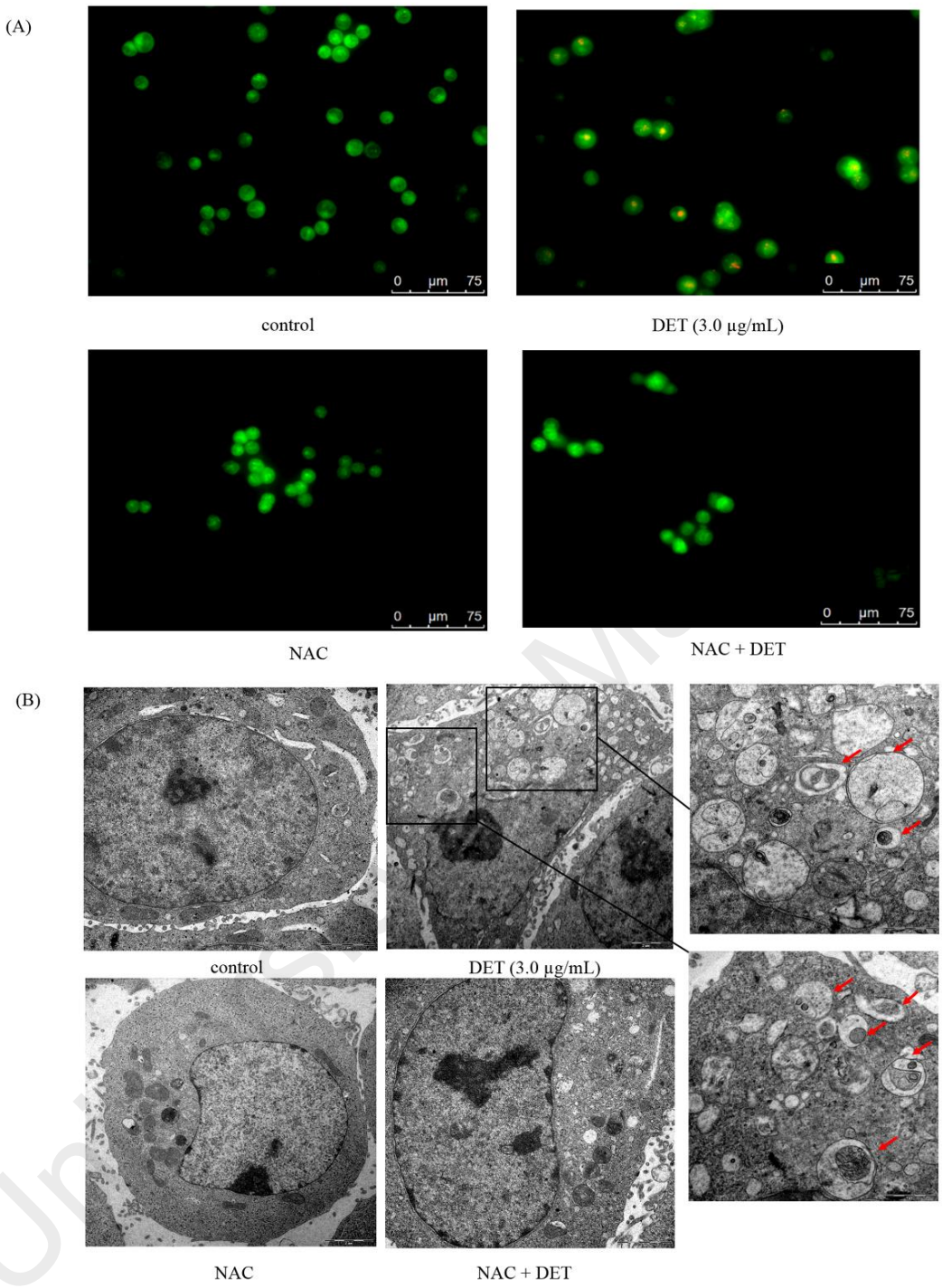


Figure 4.22: Induction of autophagy mediated by DET in HCT116 cells. HCT116 cells were preincubated with or without 1mM NAC for 1 h and then treated with DET for 24 h. (A) AVO induction upon the treatment of DET with or without the presence of NAC was determined by acridine orange staining and observed under fluorescence microscope. (B) Electron microscopy showed the ultrastructure of HCT116 colorectal cancer cells when with or without DET in the presence of NAC or without NAC for 24 h. Red arrows indicated autophagosomes including residual digested material.

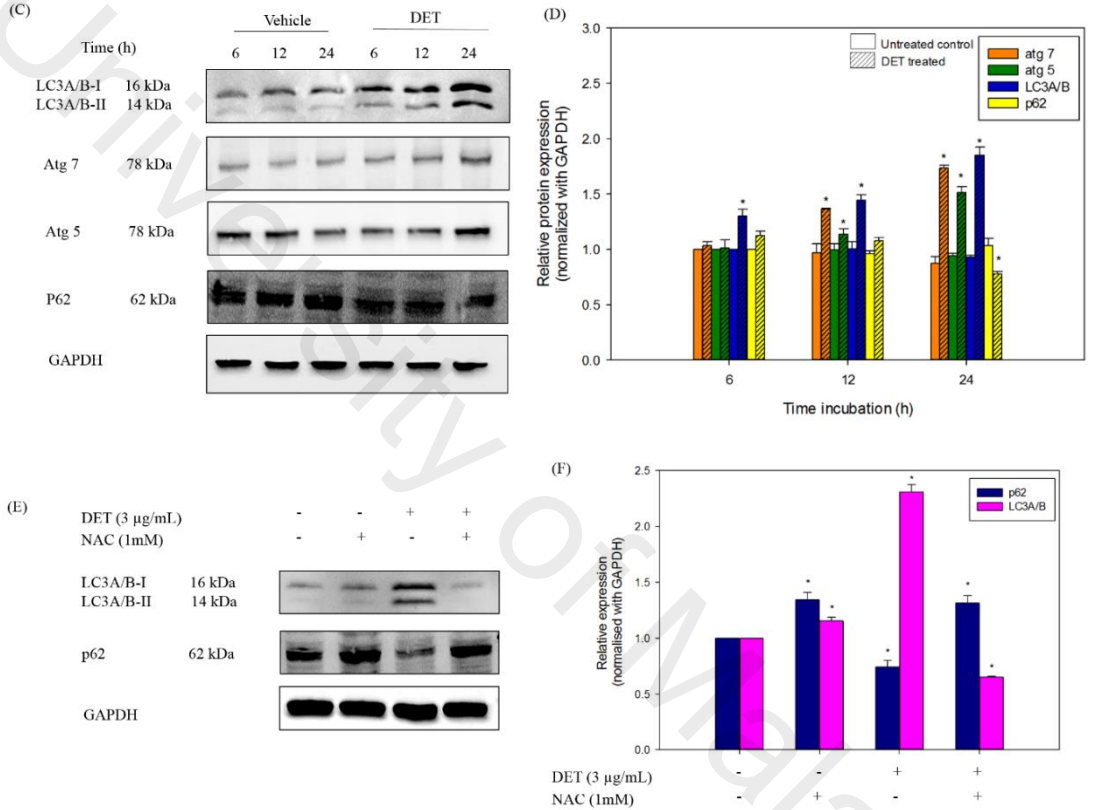


Figure 4.22, continued. (C) Representative band intensity of autophagy-related proteins including LC3, Atg 7, p62 and Atg 5. (D) Bar chart showed relative autophagy-related protein expression. (E) Representative band intensity of LC3 and p62 in presence of 1mM NAC. (F) Bar chart showed relative autophagy-related protein expression in presence of NAC.

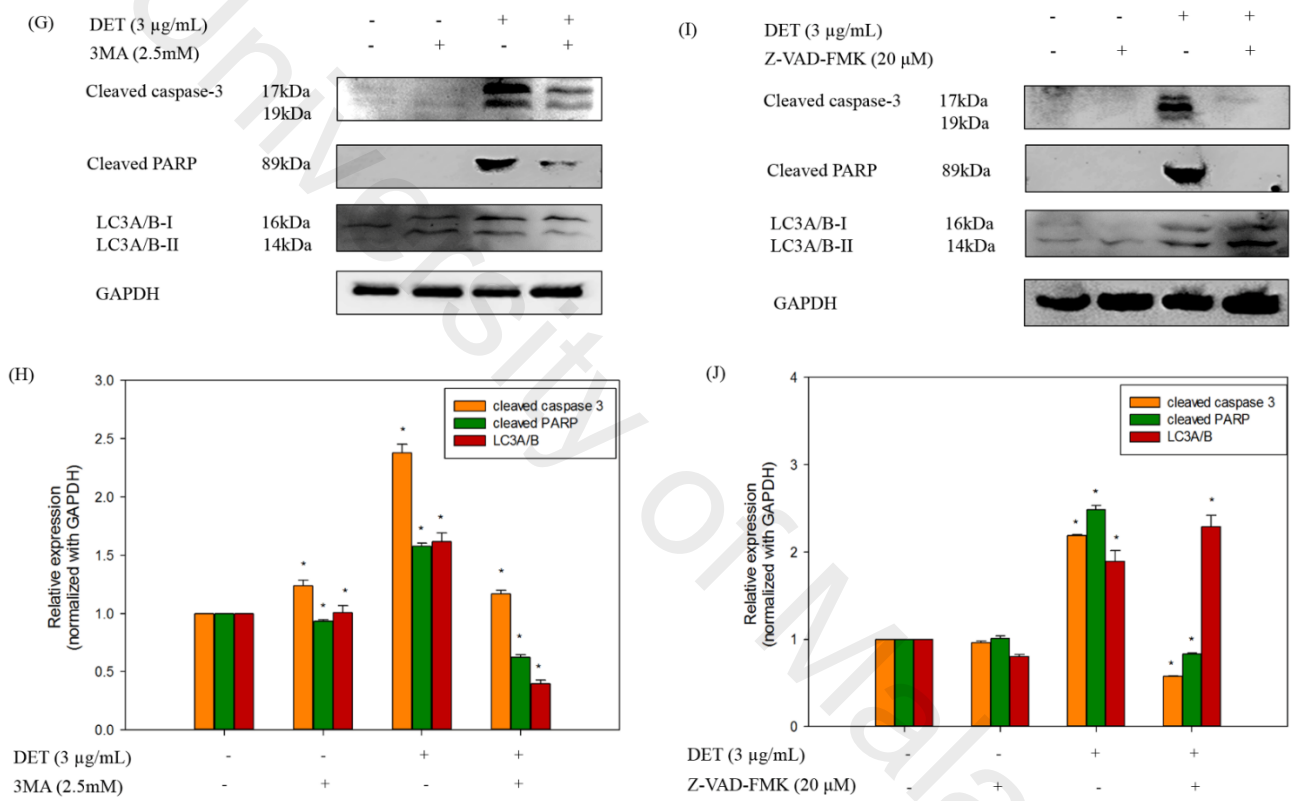


Figure 4.22, continued. Representative band intensity of cleaved caspase-3, cleaved PARP and LC3 in presence of (G) 2.5mM 3MA or (I) 20 µM z-VAD-fmk. Bar chart showed relative expression in presence with (H) 3MA or (I) z-VAD-fmk. GAPDH was used as internal control. The data presented as mean ± S.E. from three individual experiments. Asterisks denote significantly different value as compared to control (*p< 0.05).

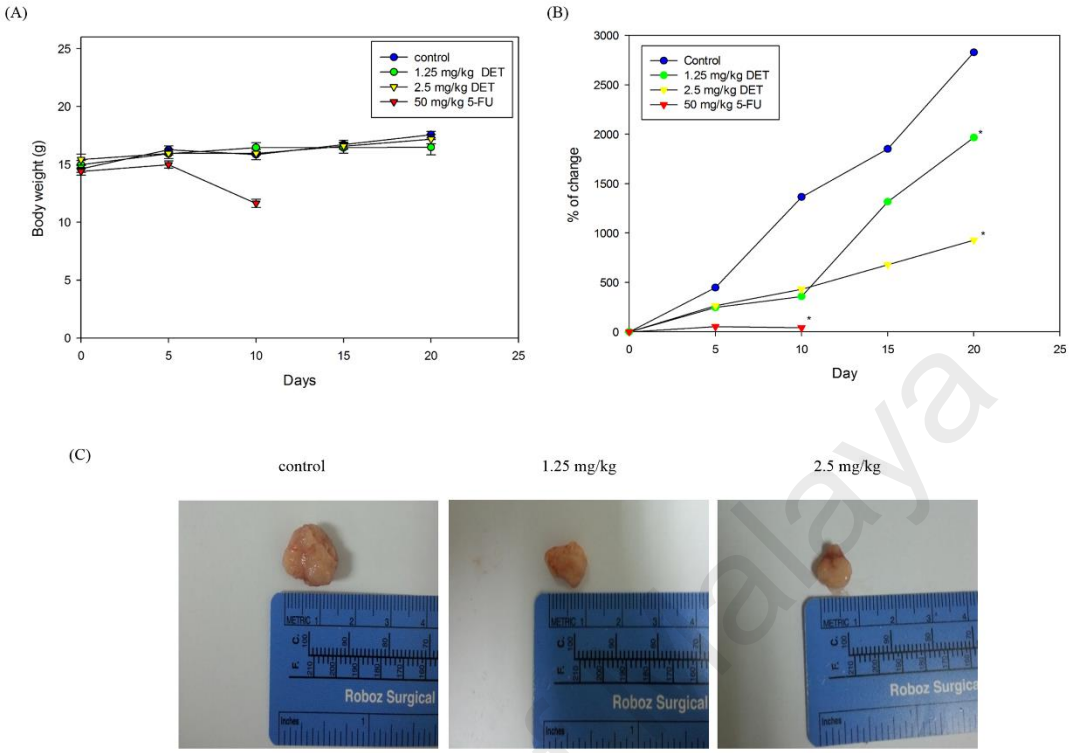


Figure 4.23: The *in vivo* anti-tumour effect of DET in HCT116 xenograft mouse model. (A) The graph illustrated the average of body weight for each group. (B) The graph represented the percentage of tumour changes for respective treatment group. (C) Representative images displayed the tumours collected from each treatment group (1.25 mg/kg and 2.5 mg/kg of DET) and vehicle control group.

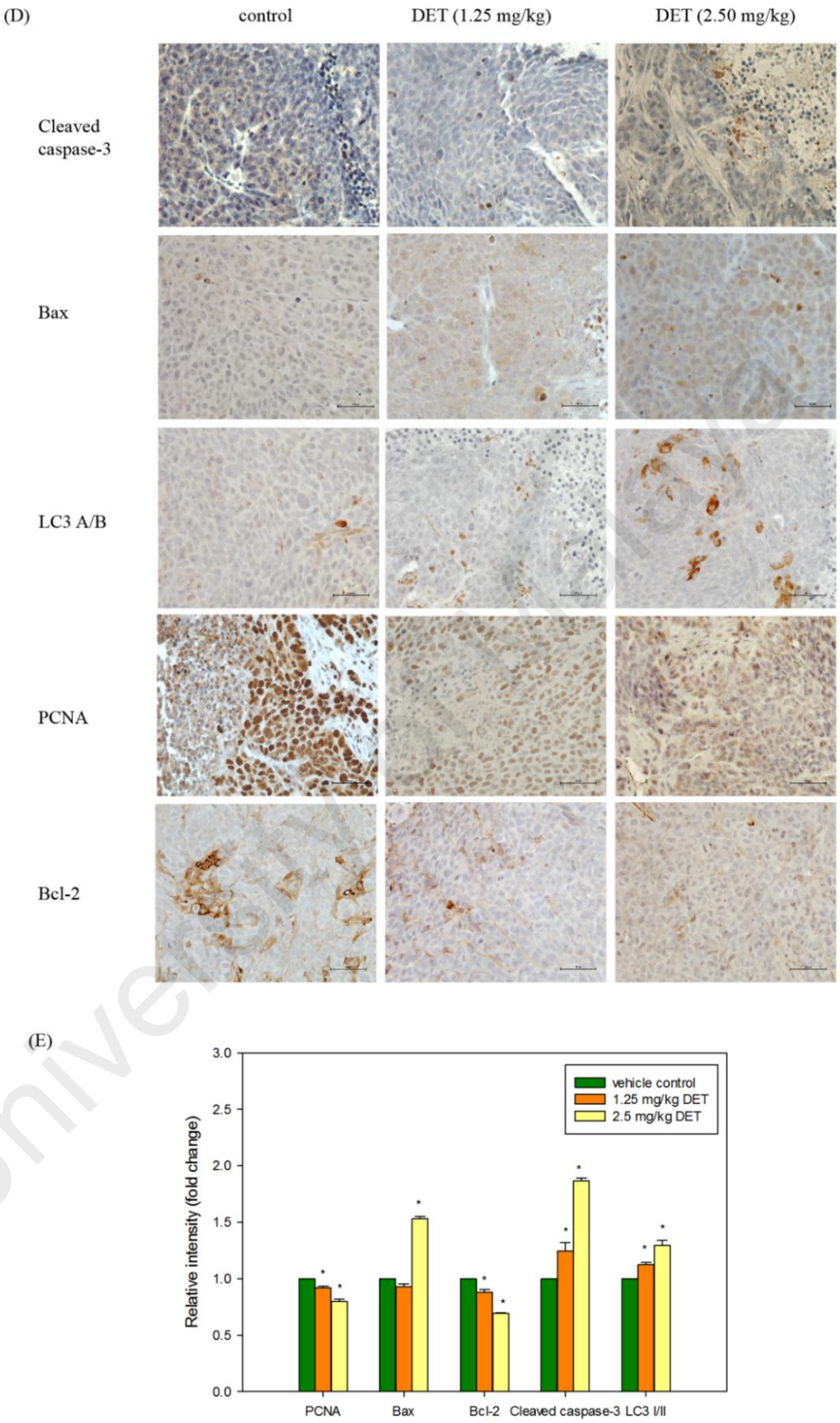


Figure 4.23, continued. (D) Representative IHC images of cleaved caspase-3, Bax, LC3 A/B, PCNA and Bcl-2 protein. (E) The bar chart revealed the relative intensity of the respective proteins. The data expressed as mean ± S.E. (n=6). Asterisks indicate significantly different value from control (*$p<0.05$).

4.25 Effects of ESEAF on the cell viability in LPS-induced BV-2 cells

ESEAF ranging from 0.5- 10 µg/mL were exposed for 2 h prior to the treatment with or without LPS in BV-2 cells for 24 h. Exposure of ESEAF alone did not revealed any apparent cytotoxic effect in BV-2 cells. However, BV-2 cell viability clearly decreased to 71.12 ± 0.73 % upon treatment with LPS alone when compared to untreated group. In contrast, pre-incubation of ESEAF followed by LPS treatment revealed that ESEAF considerably restored the cell viability as compared to LPS alone (Figure 4.24), showing the ability of ESEAF mitigating LPS-activated BV-2 cells.

4.26 Modulation of inflammatory effects by ESEAF

4.26.1 NO and PGE2 production by ESEAF

Overproduction of NO is characterized as one of the common inflammatory features (Liu *et al.*, 2002). To determine the modulation of pro-inflammatory mediator by ESEAF, the level of NO was measured in LPS-induced BV-2 cells after exposure to ESEAF (0.125, 0.25, 0.5 and 1.0 µg/mL) for 2 h. As shown in Figure 4.25(a), results depicted that LPS alone treatment group significantly intensified the NO production by 77.69 % as compared to control group. On the contrary, LPS-induced excessive NO production was dose-dependently alleviated by the treatment with ESEAF. The NO level was found declined to 36.54 ± 1.66, 35.173 ± 1.018, 22.28 ± 1.45 and 13.17 ± 0.87 nmol/mL at 0.125, 0.25, 0.5 and 1.0 µg/mL of ESEAF, respectively (Figure 4.25(a)). Next, investigation on PGE_2 production conferred by ESEAF in LPS-induced BV-2 cells was carried out. The release of PGE_2 is another inflammatory determinant of neurodegenerative diseases (Serhan & Savill, 2005). LPS alone markedly amplified the accumulation of PGE_2 as high as 10 folds in BV-2 cells. On the other hand, Figure 4.25 (b) demonstrated that ESEAF effectively attenuated the release of PGE_2 in LPS-stimulated BV-2 cells.

(A)

(B)

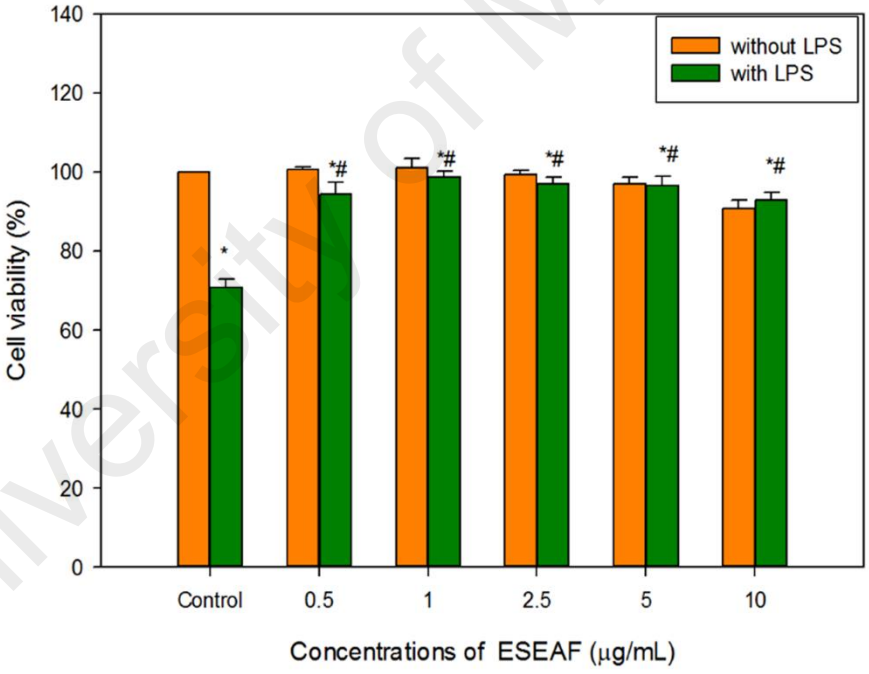


Figure 4.24: The effect of ESEAF on LPS-induced BV-2 cells. (A) Representative images indicated the plant of *Elephantopus scaber*. (B) Bar chart depicted the percentage of cell viability of BV-2 upon ESEAF treatment in presence or absence of the LPS stimulation. Data represented in mean ± S.E. (n=3). Asterisks denote values significantly different when compared to control groups (* $p < 0.05$). Hashtags denote values significantly different when compared to LPS-treated group (# $p < 0.05$).

4.26.2 Attenuation of LPS-induced iNOS and COX-2 activation by ESEAF

Next, the effect of ESEAF on the protein expression of iNOS and COX-2 were further evaluated in LPS-stimulated BV-2 cells in which detected by using western blot analysis. As depicted in Figure 4.25(c), LPS alone remarkably upregulated the protein expression of iNOS in BV-2 while ESEAF attenuated LPS-induced iNOS protein expression in a concentration-dependent manner. Furthermore, the results obviously revealed that inducible COX-2 was significantly ameliorated in an increasing concentrations of ESEAF in LPS-stimulated BV-2 cells (Figure 4.25(c)).

4.26.3 Suppression of LPS-induced p38 phosphorylation by ESEAF

Activation of p38 phosphorylation in response to the ESEAF exposure in LPS-induced BV-2 ells was determined. The phosphorylation of p38 considerably enhanced by 2.49 ± 0.07 fold when exposed to LPS alone as shown in Figure 4.25(c). Yet, when exposed to increasing concentrations of ESEAF, a significant reduction in phosphorylated p38 was observed in BV-2 cells which incubated with LPS. ESEAF markedly inactivate the phosphorylation of p38 by 1.33 ± 0.02 fold when treated with 1.0 µg/mL of ESEAF indicating the inhibitory potential of ESEAF against inflammation (Figure 4.25 (d)).

4.26.4 Inhibitory effect of ESEAF on NF-κB translocation

NF-κB transcriptional factor is found to play a cardinal role in inflammation and innate immunity by triggering inflammatory proteins including iNOS and COX-2 (Tak & Firestein, 2001). To investigate whether the attenuation of NF- κB translocation was induced by ESEAF, the nuclear and cytoplasmic of p65 NF-κB was detected by using western blot analysis. LPS alone, LPS alone induced sequestration of p65 NF-κB was found in nuclear, evidenced by the elevation in nuclear fraction of p65 protein expression (Figure 4.26(a) & (b)). Conversely, p65 was found to accumulate in cytosol upon pre-

incubation of ESEAF along with the substantial decline of nuclear p65 protein suggesting mitigation of LPS-stimulated translocation of p65.

4.26.5 NF-κB-dependent pro-inflammatory cytokine by ESEAF

Pro-inflammatory cytokines (IL-1β and TNF-α) are released upon the stimulation of NF-κB which triggers the onset of neuroinflammation (Glass *et al.*, 2010; McCoy & Tansey, 2008). Thus, to investigate the association between NF-κB and pro- inflammatory cytokines (TNF-α and IL-1β) was assessed after exposure to ESEAF in LPS-stimulated BV-2 cells. According to the results, LPS alone was found to significantly stimulate the production of TNF-α and IL-1β (Figure 4.26(c) and (d)). In contrast, the production of pro-inflammatory cytokines induced by LPS was abrogated upon pre-treatment with ESEAF indicating ESEAF mitigated inflammatory response by attenuating the secretion of pro-inflammatory cytokines. To assess the interrelation between NF-κB and the release of cytokines by ESEAF, LPS-induced BV-2 cells were pre-incubated with CAEE, a NF-κB inhibitor. Accordingly, CAEE limited LPS-induced TNF-α and IL-1β in comparison to the LPS alone and ESEAF-treated groups. In addition, a decline in TNF-α and IL-1β was detected upon treatment of ESEAF indicating that the synergistic effect of ESEAF and CAEE potentiated the anti-neuroinflammatory effect. Thus, the results suggested that ESEAF prevented pro-inflammatory cytokines release via abrogation of NF-κB in LPS-induced BV-2 cells.

4.27 Evaluation of antioxidant potential

4.27.1 Ability of ESEAF in scavenging radicals detected by DPPH assay

DPPH assay is widely utilized to screen the radical scavenging ability in order to verify the antioxidant potential of ESEAF. Antioxidant donates an electron or hydrogen to

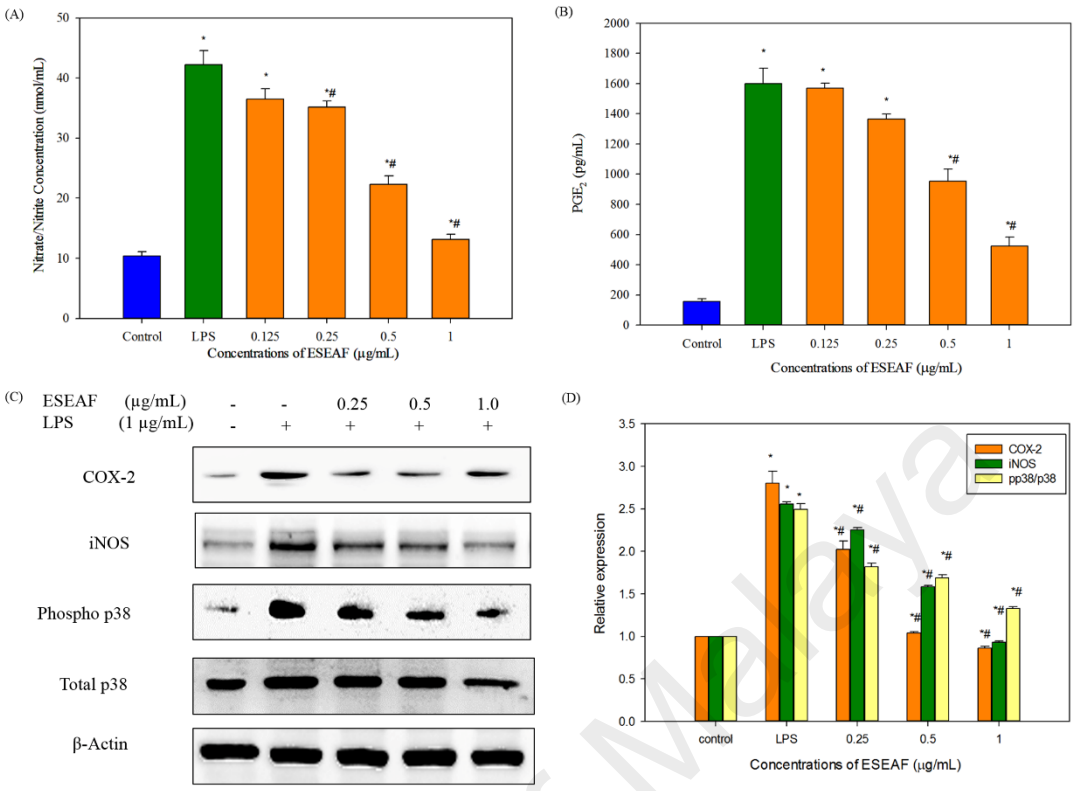


Figure 4.25: The protective effects of ESEAF against neuroinflammation in LPS-treated BV-2 cells. (A) Bar chart exhibited the generation of nitric oxide after incubation of ESEAF. (B) Bar chart showed the level of PGE_2 after incubation of ESEAF. (C) Representative band intensity of the LPS-stimmulated iNOS, COX-2, p38 and phospho-p38 upon exposure of ESEAF in BV-2 cells. B-actin as loading control throughout the experiments. (D) Bar chart demonstrated the relative expression of iNOS, COX-2 and p-p38/p38. Data represented in mean ± S.E. (n=3). Asterisks denote values significantly different when compared to control groups (* $p < 0.05$). Hashtags denote values significantly different when compared to LPS-treated group (# $p < 0.05$).

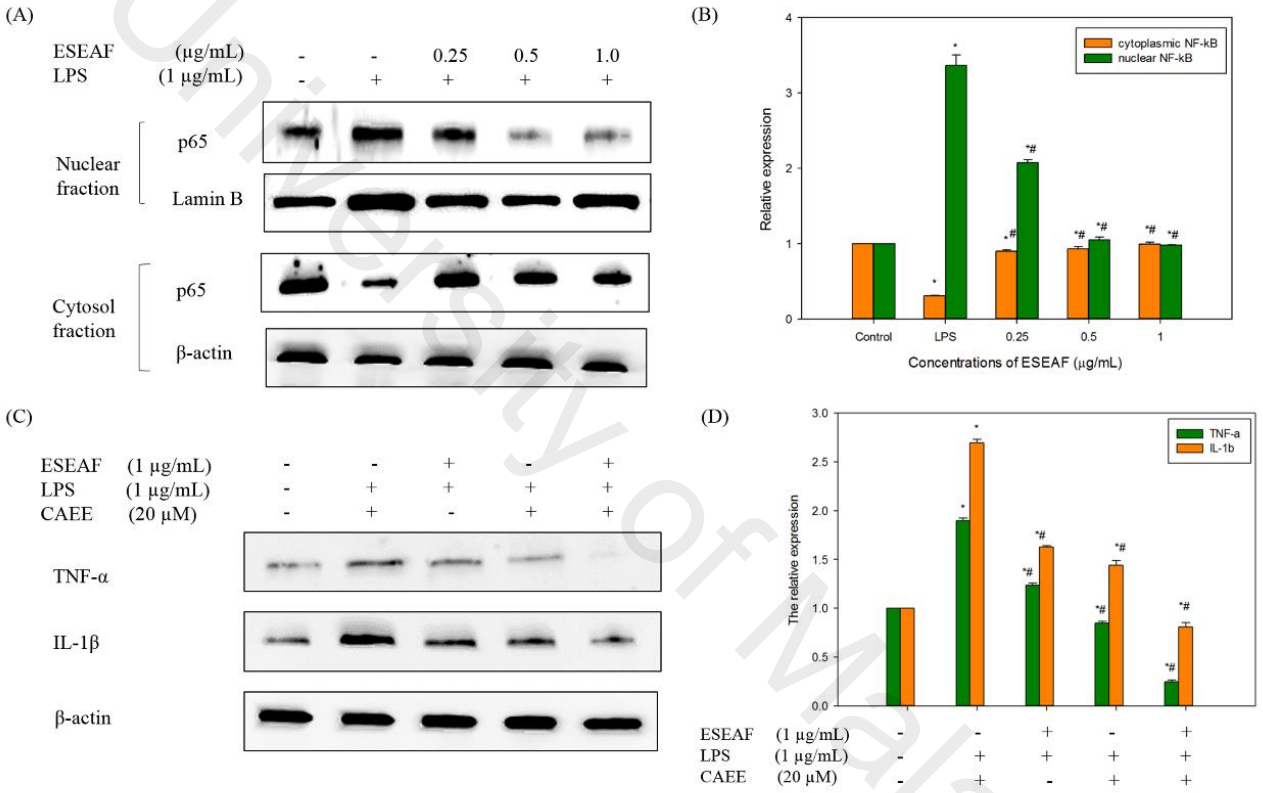


Figure 4.26: The modulatory effect of ESEAF on NF-κB and the pro-inflammatory cytokines in LPS-induced BV-2 cells. (A) Inhibition of LPS-stimulated translocation of p65 by ESEAF. (B) The bar chart depicted the relative expression of p65. (C) Attenuation of LPS-induced NF-κB-dependent pro-inflammatory cytokines by ESEAF in BV-2 cells. 20 μM of CAEE were exposed to BV-2 cells prior to the incubation of ESEAF. (D) The bar chart illustrated the relative expression of pro-inflammatory cytokines TNF-α and IL-1β. B-actin and lamin B as loading control. Data represented in mean ± S.E. (n=3). Asterisks denote significantly different in comparison to control groups (* $p < 0.05$). Hashtags denote significantly different in comparison to LPS-treated group (# $p < 0.05$).

reduce the purple stable free radical DPPH to a yellow coloured diphenylpicrylhydrazine (Molyneux, 2004). The presence of the free DPPH radicals was detected at 517nm. ESEAF effectively scavenged DPPH radicals evidenced by dose-dependent increase of radical scavenging activity as revealed in Figure 4.27(a). The inhibition of DPPH was increased from 32.41% to 85.73%. The IC_{50} value of ESEAF is 69.70 ± 0.01 µg/mL, suggesting ESEAF is a potential antioxidant.

4.27.2 Superoxide anion radical scavenging activity by ESEAF

Superoxide dismutase (SOD), an essential intracellular antioxidant defence enzyme, catalysed superoxide radicals to yield H_2O_2 and oxygen molecules (Scandalios, 1993). Therefore, the protective effect of ESEAF on SOD against radicals was examined in this study. As displayed in the Figure 4.27(b), increasing concentration of ESEAF significantly scavenged the generation of superoxide anion radical in a dose-dependent manner. Our present findings revealed that ESEAF able to protect from oxidative stress by acting as a potent superoxide anion scavenger with an IC_{50} value of 3.79 ± 0.16 µg/mL.

4.27.3 Catalase enzyme activity by ESEAF

Catalase, an endogenous antioxidant that plays key roles in neutralising H_2O_2 to water and oxygen (Brian J Day, 2009). Results showed that LPS treatment alone was found to inhibit the catalase activity (Figure 4.27(c)). Conversely, treatment with ESEAF dose-dependently enhanced the production of catalase. Upon comparison with LPS alone treated group, catalase activity was found significantly elevated by 2.69 fold when incubation with the highest concentration of ESEAF. These results proposed that ESEAF restored antioxidant potential in LPS-induced BV-2 cells.

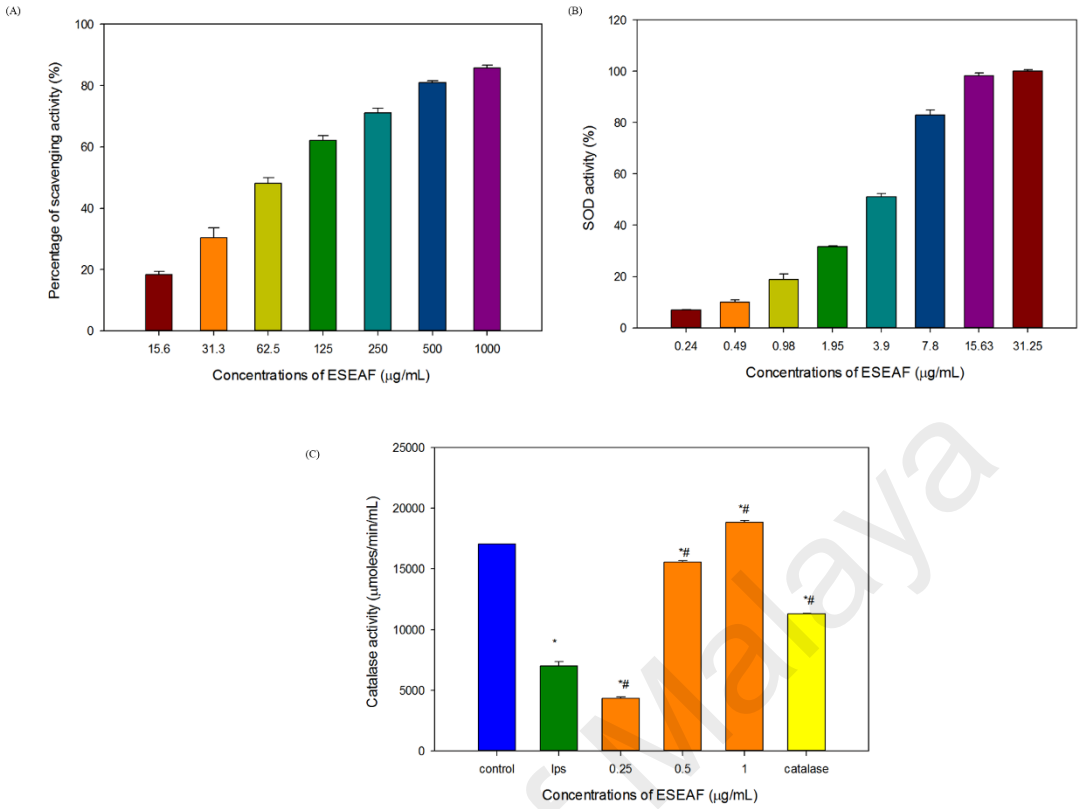


Figure 4.27: Antioxidant potential of ESEAF. (A) Bar chart demonstrated the DPPH free radical scavenging activity of ESEAF. (B) Bar chart depicted the effect of ESEAF in SOD activity. (C) Bar chart revealed the catalase enzyme activity of ESEAF. Data represented in mean ± S.E. (n=3). Asterisks denote significantly different in comparison to control groups (* $p < 0.05$). Hashtags denote significantly different in comparison to LPS-treated group (# $p < 0.05$).

4.27.4 Attenuation of intracellular reactive oxygen species level by ESEAF

To investigate the effect of ESEAF on LPS-induced oxidative stress, the generation of ROS was quantified by using DCFH-DA dye. Exposure to LPS alone significantly promoted ROS generation in BV-2 cells which is indicated by the right shift in the histogram (Figure 4.28(a)). However, LPS-induced ROS generation was restored by treatment of ESEAF. Figure 4.28(b) demonstrated that ESEAF reduced the ROS level by 0.61 ± 0.01 fold in LPS-induced BV-2 cells suggesting ESEAF a potential scavenger of reactive oxygen species.

4.27.5 Activation of antioxidant defence mechanism via Nrf2 and HO-1

It is well established that oxidative stress which triggers inflammatory response can be ameliorated via the activation of Nrf2/HO-1 pathway (A. Paine *et al.*, 2010; van Muiswinkel & Kuiperij, 2005). To elucidate the involvement of ESEAF on the antioxidant defence mechanism, Nrf2 and HO-1 proteins were analysed by using western blot analysis. Our findings revealed that the cytoplasmic fraction of Nrf2 was gradually reduced in a concentration-dependent manner upon ESEAF treatment in LPS-stimulated cells (Figure 6.5(c)&(d)). These findings suggested that ESEAF induced the translocation of Nrf2 from cytoplasm to nucleus. Thereafter, downstream effectors of Nrf2 transduction signalling which is HO-1 was proceeded for further investigation by using western blot analysis. ESEAF was found to prominently upregulate HO-1 protein expression in LPS-stimulated BV-2 cells in comparison to LPS treatment alone (Figure 6.5(c)&(d)). Our findings indicated that ESEAF exerted its protective effect by stimulating Nrf2 via HO-1 in LPS-stimulated BV-2 cells.

4.27.6 The effect of ESEAF on gross pathology and blood biochemistry parameters

Haematoxylin and eosin staining were used to verify the histopathological condition of

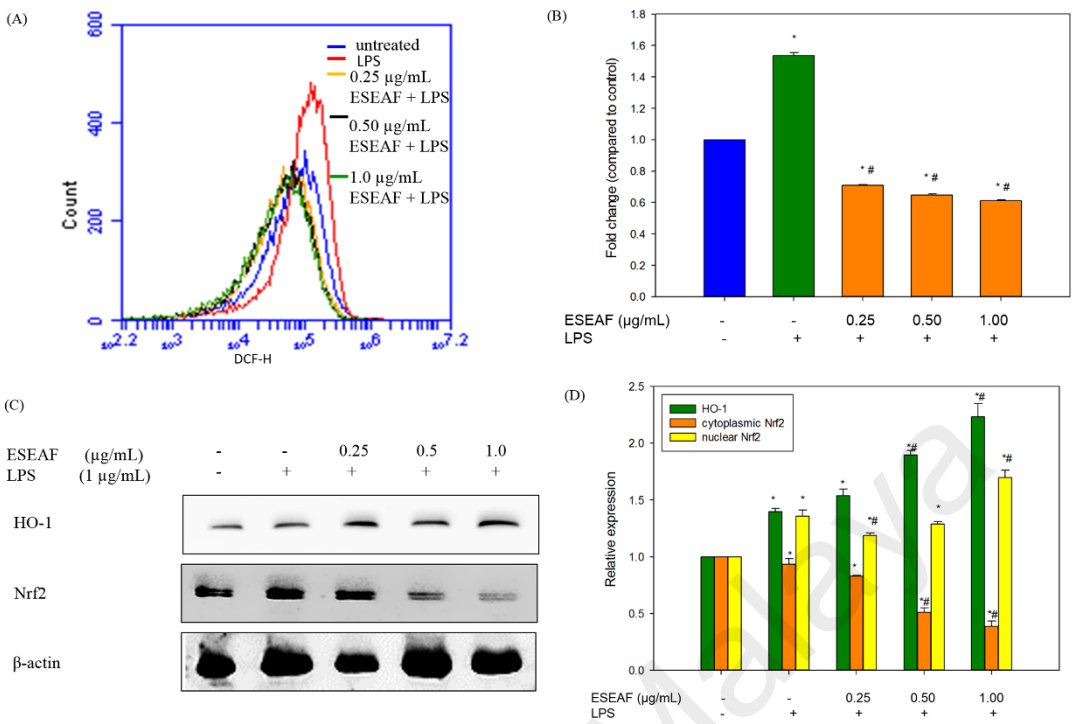


Figure 4.28: Antioxidant defence mechanism against oxidative stress via Nrf2/HO-1 signalling by ESEAF in LPS-induced BV-2 cells. (A) The histogram represented the level of ROS elicited by ESEAF. (B) Bar chart depicted the fold change of the LPS-induced generation of intracellular ROS in ESEAF treated BV-2 cells. (C) Representative band intensity of the antioxidant defence-related proteins, Nrf2 and HO-1. B-actin as loading control throughout all experiments. (D) Bar chart indicated the relative expression of Nrf2 and HO-1. Values represented in mean ± S.E. (n=3). Asterisks denote significantly different in comparison to control groups (* $p < 0.05$). Hashtags denote significantly different in comparison to LPS-treated group (# $p < 0.05$).

the organs. Gross necropsy findings demonstrated that absence of atypical histopathological lesions were detected in all the main organs as shown in Figure 6.6. Subsequently, clinical biochemical parameters in the serum examined by the autoanalyser were recorded in Table 6.2. Measurement of clinical biochemical parameters including the serum levels of CREA, TBIL, albumin, ALT, AST and ALP has no any significant differences were noticed in ESEAF treated group as compared to the control group suggesting ESEAF treatment did not bring any adverse effects.

4.27.7 Acute toxicity and symptoms

The acute toxicity test demonstrated that upon oral administration of 2000mg/kg of ESEAF, no mortality and no symptoms of toxicity or behaviours were observed in all animals throughout the 14 days observation.

4.27.8 Organ weight/BW coefficients

The body weight on day 0 and day 14 were recorded. After all the mice were sacrificed, the organs such as liver, spleen, kidney, lung and heart were collected and weight of organs was recorded. There are no statistically significant change in body weight was observed in the treated mice as compared to the control group. However, over a period of 14 days, progressive body weight gain were noticed in both groups of mice. Table 6.1 summarized the coefficients of kidney, liver, spleen, heart and lung to body weight which are presented as milligrams (wet weight tissues)/g (body weight) which suggesting no apparent differences in both treated and untreated groups.

4.27.9 Histopathological detection and blood biochemical analysis

Gross examination during autopsy and histopathological lesions were not observed in any of the main organs which stained with haematoxylin and eosin (Figure 4.6). Biochemical

parameters in the serum detected by the autoanalyser were listed in Table 4.7. No significant differences were discovered in the serum levels of CREA, albumin, TBIL, ALP, AST and ALT in ESEAF treated mice as compared to the control group.

Table 4.6: Organ weight/BW coefficients (Mean ± SE)

Groups	Body weight (g)		Liver	Kidney	Spleen	Lung	Heart
	Before	After					
Control	24.72±0.86	27.94±0.59	42.91±1.56	13.58±0.60	3.56±0.07	8.54±0.71	4.28±0.68
Treated 2000mg/kg	25.2±0.75	26.88±0.81	46.20±0.77	12.69±0.97	3.73±0.11	11.88±1.30	4.51±0.82

Table 4.7: Biochemical parameters for each respective group of blood serum

Biochemical parameters	Control group	Treated group (2000mg/kg)
Creatinine (CREA)	10.86±0.40	9.83±0.31
Albumin	25.71±0.18	25.33±0.67
Total bilirubin (TBIL)	0.71±0.42	0.33±0.21
Alanine phosphatase (ALP)	99.29±5.02	97.00±4.31
Aspartate aminotransferase (AST)	72.57±12.15	111.50±29.63
Alanine aminotransferase (ALT)	17.86±2.35	23.83±3.17

Data represented as mean ± SE.

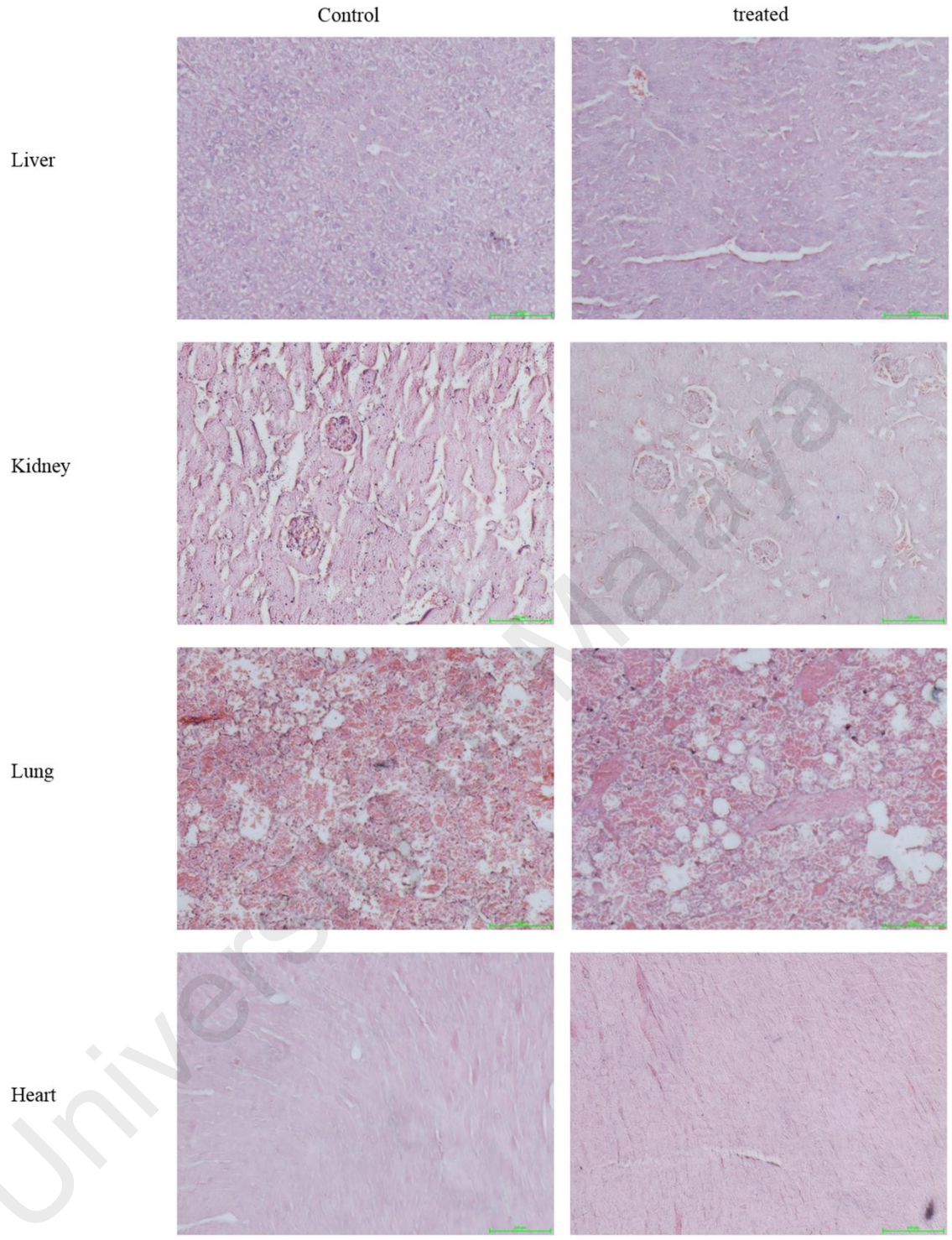


Figure 4.29: Gross histopathological assessment of all the collected organs comprising liver, heart, kidney, lung and spleen when oral administration with 2000mg/kg ESEAF as compared to untreated group. Magnification: 200x

CHAPTER 5: DISCUSSION

Plants have been the basis for the treatment of various dreaded diseases and still remained as alternatives for cancer treatment. This is accordance with WHO reports, approximately 65% of the population predominantly relied on the plant-derived traditional medicines for their primary care (Cragg & Newman, 2013; Farnsworth *et al.*, 1985). In the present study, apoptosis inducing effects of *E. scaber* was investigated in human colorectal HCT116 cells. Based on our studies, ESEAF exhibited as the most effective fraction to suppress the proliferation of HCT116 cells and HT-29 human colorectal cancer cells among the crude extract and different fractions of *E. scaber* via *in vitro* cytotoxic MTT assay. For the first time, our present study demonstrated that *E. scaber* exerted the most potent cytotoxicity against HCT116 cells. Conversely, ESEAF did not exert cytotoxic effect against normal colon cells. Thus, ESEAF and HCT116 cells were subjected to further investigation on apoptosis.

Apoptosis is one of the fundamental cellular demise to maintain tissue homeostasis and development which contribute in various pathogenesis. It is the most common approach for the development of therapeutic drugs against cancer. Apoptosis is generally distinguished by various morphological features such as cell shrinkage, membrane blebbing, DNA fragmentation and nuclear chromatin condensation along with biochemical changes such as externalization of phosphatidylserine and caspase activation (Elmore, 2007; Hotchkiss *et al.*, 2009). To gain further insight on the apoptosis inducing effects of ESEAF, the biochemical and morphological changes which are the common hallmarks of apoptosis were determined. Hoechst 33342 is a membrane-permeant dye that preferentially binds to AT-rich regions in DNA whereas propidium iodide (PI) is a DNA specific dye which impermeable to live cell's membrane (Martin *et al.*, 2005). Both of the fluorescence dyes are used for morphological detection to distinguish the live cells

and apoptotic cells. In our study, treatment of ESEAF was found to induce atypical nuclear morphological alterations which manifested several features including nuclear shrinkage, chromatin condensation, DNA fragmentation as well as late apoptosis upon double staining by Hoechst 33342 and PI, indicating the induction of apoptosis by ESEAF in HCT116 cells. In addition, the ESEAF-induced apoptosis was further supported by the occurrence of DNA fragmentation via TUNEL assay. Furthermore, externalization of phosphatidylseine (PS), is regarded as one of the biochemical hallmarks which can distinguish healthy, early apoptotic, late apoptotic and necrotic cells by Annexin V and PI staining. Annexin V is a probe that preferentially binds to PS, which is translocated to outer membrane during the early apoptosis owing to the asymmetry of cell-surface phospholipid (Zwaal *et al.*, 2005). As shown in our results, ESEAF induced the loss of plasma membrane asymmetry which evident by the increase of Annexin positive cell populations indicating increasing phosphatidylserine is externalized to outer surface of plasma membrane. This collective findings suggested that the ESEAF exerts apoptosis-inducing effects in HCT116.

Mitochondria are essential organelles in energy production which also an integral nodal of intrinsic apoptotic pathway via MOMP (Martel *et al.*, 2012; Martinou & Youle, 2011). MOMP is tightly orchestrated by pro- and anti-apoptotic Bcl-2 family proteins (Martinou & Youle, 2011). The imbalance of pro-and anti-apoptotic Bcl-2 tilts towards survival whereby render cells more resistance to chemotherapeutic drugs or stimuli and hence promote tumour formation and progression (Reed, 1994). Therefore, ratio of pro- and anti-apoptotic Bcl-2 proteins is dictated as an essential cellular fate determinant. An increase of Bax/Bcl-2 ratio render cells more susceptible to apoptosis by increasing MOMP eventually resulted in impairment of mitochondrial function. Ample studies has reported that dysfunction of mitochondria is correlated to MOMP and resulted in loss of mitochondrial membrane potential ($\Delta\Psi m$) which is classified as one of the early apoptosis

hallmarks (Salvioli *et al.*, 2000). This phenomenon in turn initiates apoptosis which is regarded as the "point of no return" (Green & Kroemer, 2004). In the present study, a time-dependent upregulation of Bax expression along with an increase of Bax/Bcl-2 ratio was detected upon the treatment of ESEAF in HCT116 cells which contributes in evoking a time-dependent collapse of $\Delta\Psi m$. This collapse is attributed to the conformational changes of pro-apoptotic protein, Bax which promotes the formation of pores in mitochondria, consequently unleash apoptotic molecules such as cytochrome c and thus amplify the apoptotic signals via caspase cascades (Bratton & Salvesen, 2010; Tait & Green, 2013; Zou *et al.*, 2003).

Caspases, a family of cysteine proteases encompassing caspase-3, -7, -8 and -9 which is closely associated with the apoptosis cell death machinery (Degterev *et al.*, 2003). It is well documented that both initiator caspase-8 and caspase-9 regulates extrinsic pathway and intrinsic pathway, respectively, which further activates executioner caspase-3 or -7. For intrinsic pathway, loss of $\Delta\Psi m$ results in the release of cytochrome c further facilitates the formation of apoptosome consisting Apaf-1 and pro-caspase-9 which in turn activates the cleavage of caspase-3. Additionally, caspase-3 is known to responsible for the cleavage of PARP prior to the induction of apoptosis (Fan *et al.*, 2005). PARP contributes in several biological events such as apoptosis, transcription and cell cycle regulation and maintenance of genome integrity. This is in lined with our present findings that post treatment with ESEAF induced the activation of caspase-9 and -3/7 accompanied with the cleavage of PARP in HCT116 cells. The increase of PARP cleavage is dictated with the accumulation of DNA fragmentation whereby are regarded as indicative of caspase activation (Bressenot *et al.*, 2009) which in accordant with our nuclear morphology observations and the increase of TUNEL positive cells. These collective evidences suggested that ESEAF induced cell death via mitochondrial-mediated apoptosis pathway in HCT116 cells.

Excessive generation of ROS has been closely associated with tumourigenesis and progression. Paradoxically, empirical evidences has clearly suggested that aberrant generation of ROS beyond the toxicity threshold may lead to apoptosis in various cancer cells. Therefore, great interest has attracted among the scientist by using this pro-oxidant therapy as an alternative strategy for the cancer therapy (Galadari *et al.*, 2017; Pelicano *et al.*, 2004). Upon exposure of ESEAF, accumulation of ROS was noticed in HCT116 cells which might owing to the release of free radical during cytotoxicity. It is well established that the onset of apoptosis is preceded by the stimulation of ROS. Thus, the accumulation of ROS was correlated with ESEAF-induced apoptosis in HCT116 cells. This is evidenced by the attenuation of ROS production which resulted in restoration of cell viability by the pre-treatment with ROS scavenger NAC. Mitochondrial-mediated apoptosis galvanized by caspase-9 and -3/7 eventually leads to DNA fragmentation via cleavage of PARP. Interestingly, cleavage of PARP which is one of the apoptotic biomarkers (Duriez & Shah, 1997) was found to be prevented in presence of NAC, supporting the notion that suppression of apoptosis is associated with ROS. These findings are corroborated with several studies of ROS-inducing agents, for instance, resveratrol (Juan *et al*., 2008) and flavokawain B (Kuo *et al*., 2010) exerted cytotoxic effects and prevented the ROS-dependent intrinsic pathway in colorectal cancer cells in the presence of NAC. Therefore, these results provide substantial evidences implicating ESEAF elicited ROS-dependent mitochondrial-mediated apoptosis in HCT116 cells.

P53 is widely recognized as a tumour suppressor which contributes in various fundamental cellular events including apoptosis and cell cycle arrest (Vousden & Prives, 2009). This tumour suppressor gene initiates apoptosis via mitochondria-mediated apoptosis pathway by modulating the Bax/Bcl-2 ratio (Green & Kroemer, 2004). It is well documented that transcription factor p53 mediates apoptotic signal by binding to the apoptosis responsive genes to promote the pro-apoptotic genes such as Bax (Chipuk *et*

al., 2004; Oren & Bartek, 2007). This was corroborated with our present findings that ESEAF induced an activation of p53 accompanied by an increase of Bax which resulted in an elevation of Bax/Bcl-2 ratio. In another words, activation of p53 preceded upregulation of Bax to facilitate apoptosis via mitochondria by ESEAF in HCT116 cells.

Several of phytochemical constituents which were identified in *E. scaber* including sesquiterpene lactones, steroids and triterpenoids were discovered in ESEAF by GC-MS analysis. Presence of these phytochemicals were reported to exert various biological activities. Among these, *E. scaber* was found to contain a rich source of sesquiterpene lactones which has been highlighted in a number of studies with its cytotoxicity and anticancer potential against several cancer cell lines including chronic myeloid leukemia, nasopharyngeal, breast, lung adenocarcinoma and mammary cancer (Ichikawa *et al.*, 2006; Kabeer *et al.*, 2013; Kabeer *et al.*, 2014; Lee & Shyur, 2012; Su *et al.*, 2011). Furthermore, lupeol also exhibits apoptotic effects in breast cancer and human epidermoid carcinoma (Pitchai *et al.*, 2014; Prasad *et al.*, 2009). Accordingly, the presence of sesquiterpene lactones and triterpenoids such as deoxyelephantopin, isodeoxyelephantopin and lupeol in *E. scaber* may be responsible for the observed apoptosis-inducing effects in HCT116 cells which corroborated with the apoptotic findings reported earlier.

In summary, an accumulation of ROS was elicited by ESEAF further trigger p53 activation leading to the collapse of mitochondria membrane potential via the modulation of the Bax/Bcl-2 ratio, activation of caspase-9 and -3/7. This consequently induced the cleavage of PARP which in turn caused the fragmentation of DNA and ultimately apoptosis in HCT116 cells. Taken together, the proposed apoptosis mechanism mediated by ESEAF was illustrated in Figure 5.1. For the first time, our present study delineated that ESEAF suppressed HCT116 cell growth by eliciting caspase-dependent

mitochondrial-mediated apoptosis via oxidative stress. Thus, ESEAF may be a promising and novel integrative and complementary medicine for the treatment of HCT116 human colorectal cancer cells. Nevertheless, further studies are needed to identify and isolate the bioactive constituents from ESEAF which may be attributed to the anticancer activity and to establish molecular mechanisms.

Sesquiterpene lactones are terpenoids which are ubiquitously found in the family of Asteraceae and exerts a notable spectrum of pharmacological activities including gastroprotective, anti-inflammatory, anticancer, antimalarial, antimigraine and analgesic (Giordano *et al.*, 1990; Lyss *et al.*, 1998; Prehn & Krieglstein, 1993; Wesolowska *et al.*, 2006; Wong & Menendez, 1999; Zhang *et al.*, 2005). Owing to the presence of a rich source of sesquiterpene lactones in *E. scaber*, enormous interest has raised among the researchers on the potential anticancer activities (Amorim *et al.*, 2013; Hiradeve & Rangari, 2014). Deoxyelephantopin (DET) from *E. scaber* emerges as one of the most promising phytoconstituents for the treatment of cancer (Hiradeve & Rangari, 2014). In the present study, DET was identified as the bioactive phytochemical which exerted the apoptosis inducing effect and cell cycle arrest followed by elucidation on its underlying mechanisms in HCT116 cells.

Our previous findings demonstrated that ethyl acetate fraction of *E. scaber* exhibited anticancer effect against HCT116 human colorectal cancer cells (Chan *et al.*, 2015). Based on the results obtained, ethyl acetate fraction of *E. scaber* was proceeded for isolation and identification of the bioactive compound(s) which are responsible for the cytotoxic effects via bioassay-guided approach. Accordingly, bioassay-guided isolation led to the identification of two sesquiterpene lactones, DET and isoDET and that DET potentially suppressed the proliferation of HCT116 cells with lower IC_{50} value than isoDET. The cytotoxic effect of DET was also studied on normal colon cells.

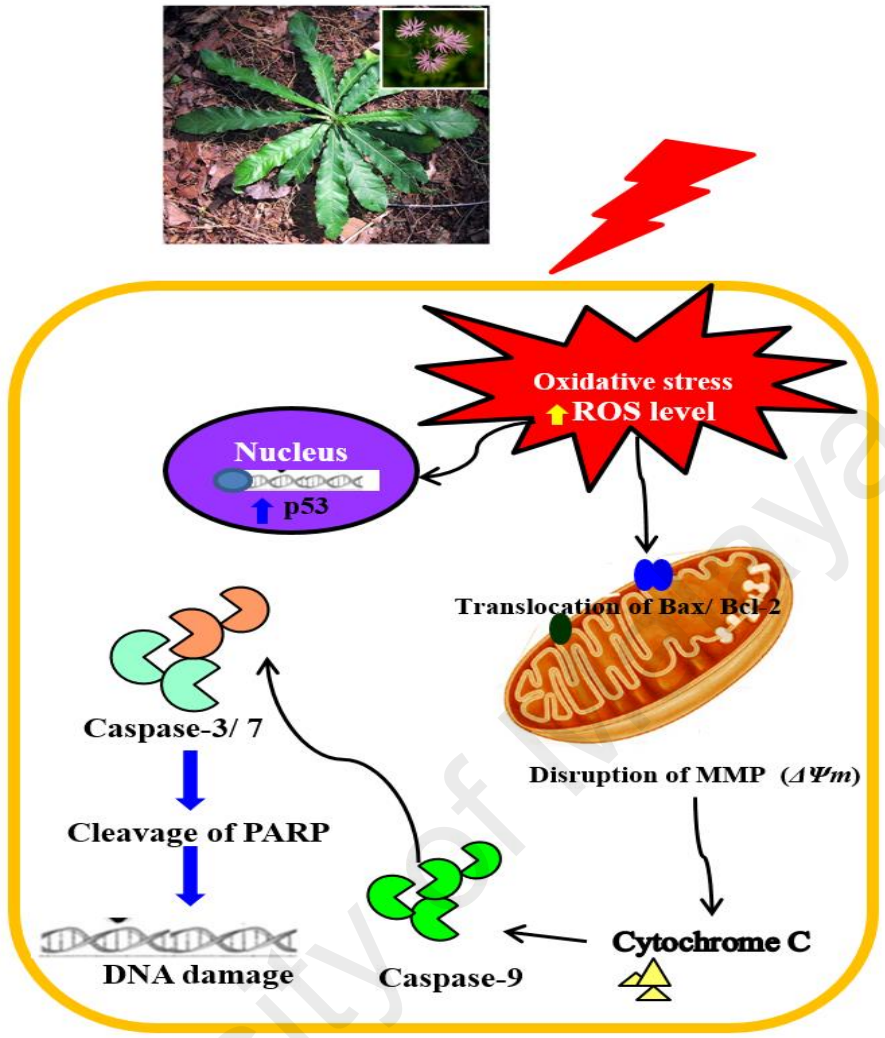


Figure 5.1: Schematic illustration displayed the apoptosis underlying mechanism by ESEAF in HCT116 cells. ESEAF suppressed HCT116 cell growth by activating cleavage of caspase-9 and caspase-3/7 followed by cleavage of PARP. This further resulted in DNA damage and eventually induced apoptosis via oxidative stress.

Interestingly, DET revealed a considerably lower cytotoxic effect in CCD841 CoN normal colon cells where the selectivity index is more than 30. Based on the findings, DET was a more potent growth inhibitor than isoDET, hence, it was proceeded for further explorations on the underlying mechanisms.

Decreased cell viability could be as a consequence of reduced proliferation or increased cell death, via several cell death modalities such as apoptosis, autophagy and necrosis (Nikoletopoulou *et al.*, 2013). Apoptosis is an evolutionarily conserved event which is distinguished by morphological and biochemical hallmarks including nuclear shrinkage, chromatin condensation, phosphatidylserine externalization, DNA fragmentation, depolarisation of mitochondria membrane potential and activation of caspase (Wong, 2011). In the present study, Hoechst 33342/ PI staining displayed a pronounced increase in nuclear morphological changes encompassing DNA fragmentation, nuclear shrinkage and chromatin condensation in dose-dependent manner upon treatment with DET in HCT116 cells. The disintegration of asymmetrical plasma membrane instigated the translocation of phosphatidylserine to the outer leaflet of plasma membrane representing the early event of apoptosis which can be detected by Annexin V-FITC/PI assay (Vermes *et al.*, 1995). In the early apoptosis, the cell membrane remains intact whereas during late apoptosis and necrosis, asymmetrical integrity of plasma membrane is perturbed and permeable to impermeable propidium iodide fluorochrome (Vermes *et al.*, 1995). Based on collective evidences, the growth inhibitory effect of DET in HCT116 cells was attributed to the induction of apoptosis which is implied by the morphological analysis and phosphatidylserine externalization.

Activation of caspases is a highly conserved mechanism to commit cellular suicide which is one of the approaches in combating cancer. Executioner caspase-3 is an integral arbitrator participating in the induction of apoptosis as it is accountable for the proteolytic

degradation of several substrates including PARP and lamin. Proteolytic degradation of PARP by caspase-3 results in cleaved PARP whereby it plays important roles as an apoptotic amplifier in facilitating the cellular disintegration and DNA fragmentation to block DNA repair cycles (Lavrik *et al.*, 2005; Li & Darzynkiewicz, 2000). Thus, our findings indicated that DET induced apoptosis accompanied by a dose-dependent caspase-3 activation and cleavage of PARP. In line with the notion, the cleavage of PARP was accompanied by the increased occurrence of DNA fragmentation in DET-treated cells. Accordingly, these findings clearly suggested that DET triggered cell death via apoptosis in HCT116 cells.

Evolution of cancer cells occurs as a consequences of intervening normal cell cycle regulation (Gabrielli *et al.*, 2012). Regulation of cell cycle is one of the vital approaches in targeting cancer (Urrego *et al.*, 2014). Cell cycle checkpoints prevent the genomic DNA from errors during replication and chromosome aggregation (Nyberg *et al.*, 2002). It has been reported that cytotoxic phytochemicals could act as cell cycle modulators by initiating cell cycle arrest at specific checkpoints and lead to the execution of apoptosis as potential strategy for cancer therapy (Ahmad *et al.*, 2000; Li *et al.*, 2016). Notably, the essential phases of the cell cycle are the S phase, the emergence of proper DNA replication and G2/M phase, the cell division into two daughter cells (Williams & Stoeber, 2012). In response of DNA damage, cell cycle arrest at S and G2/M phases allows DNA repair to prevent further replication and mitosis (Bartek *et al.*, 2007). The present study revealed that treatment with DET resulted in cell cycle arrest at S and G2/M phase in HCT116 cells. In accordance with the previous study, the growth inhibitory effect of DET was via suppression of cell cycle progression at both S and G2/M in human CNE nasopharyngeal cells (Su *et al.*, 2011). Apart from this, cell cycle progression is suggested to closely associate with apoptosis in which the blockade of cell cycle progression may ultimately lead to apoptosis in response to DNA damage (Visconti *et al.*, 2016).

Interestingly, DET induced an increase in cell populations at sub G1 phase in HCT116 cells which is indicative of apoptotic cells accumulation. The accumulation of the apoptotic cells was substantiated by the escalating increase of the apoptotic determinants such as cleaved caspase-3 and cleaved PARP which was previously detected. The conclusive evidence suggested that the proliferation of HCT116 cells was prevented via the inhibition of cell cycle progression and the induction of apoptosis by DET.

The p53 has been widely recognized as tumour suppressor that mediates several mechanisms such as cell cycle arrest and apoptosis to preserve genome integrity in response to various stress stimuli and DNA damage (Bieging *et al.*, 2014). Our results demonstrated that DET increased the activation of p53 in HCT116 cells indicating that p53 was closely related to the apoptosis induction and cell cycle arrest. Cell cycle machinery is a tightly regulated integral event which is positively regulated by cyclin-dependent kinase (CDK)/cyclins complexes and negatively regulated by CDK inhibitory proteins. The transcription factor p53 encodes and regulates a wide variety of genes including p21, cyclin-dependent kinase inhibitor (Deiry *et al.*, 1993; Harper *et al.*, 1993). Exploitation of p21 as a potential target of chemotherapeutic drugs was found to be able to disrupt tumourigenesis by activating the S and G2/M checkpoints of the cell cycle in cancer cells via p53 activation upon DNA damage (Bunz *et al.*, 1998; Dotto, 2000; Fischer *et al.*, 2016; Ogryzko *et al.*, 1997; Roninson, 2003). For instance, oridonin inhibited the cancer cell growth via cell cycle arrest at S phase through activation of p53 and p21 (Cui *et al.*, 2007). This is corroborated by our present findings which demonstrated that cell cycle arrest is putatively activated by DET via p53 leading to a significant upregulation of p21 in HCT116 cells.

The cyclin-dependent kinase inhibitor, p21, hinders cell cycle progression by directly binding to different CDK/cyclin complexes at different phases (Deiry *et al.*, 1993). A

concerted action of cyclin A, cyclin E, cyclin D, CDK4, CDK6, and CDK2 is required in the initiation of G1/S transition (Gottifredi *et al.*, 2004). Conversely, cyclin A/CDK2 promotes the cell cycle progress from S to G2 phase followed by the entry of mitosis which is attributable to the cyclin B/CDK1 complex as a crucial modulator of G2/M checkpoint (Lim & Kaldis, 2013; Schutte *et al.*, 1997). It is well established that cyclin B, cyclin A, CDK1 and CDK2 facilitate G2/M cell cycle progression (Lim & Kaldis, 2013). Thus, inhibition of cyclins and CDKs prevents the progression of cell cycle by various phytochemicals. For instance, gallic acid, commonly found in tea leaves, reduced cyclin A, CDK2, cyclin B1 via p21 in MCF-7 human breast cancer cells (Hsu *et al.*, 2011). A decline in cyclin B1, cyclin D1, cyclin E and CDK2 and cyclin A was observed upon exposure to resveratrol and hinokitiol, in SW480 human colorectal adenocarcinoma cells and HCT116 human colon cancer cells, respectively, which led to S-phase cell cycle arrest (Joe *et al.*, 2002; Lee, 2013). Present findings revealed that DET initiated S and G2/M phase arrest via downmodulation of CDK2, CDK4 cyclin A2, cyclin D1, cyclin E2 and cyclin B1 protein expression. The increase in phospho-p53 and p21 attenuated the activation of cyclin E and cyclin A/CDK2 which are required for progressing G1 into S phase.

Deoxyelephantopin, a major natural compound derived from *E. scaber* has been shown to possess potent anticancer effect against numerous human cancer cell lines. Mounting interest resulted in the exploration of DET as a drug lead owing to its multi-functional roles including anti-migration, anti-invasion anti-angiogenesis, anti-inflammation and anti-tumour via multiple signalling transductions. It has been previously reported that DET suppressed proliferation of cancer cells via several signalling transductions encompassing cell cycle, apoptosis, NF-κB, signalling transducer and activator of transcription 3 (STAT) and PI3K/Akt pathways. Hence, multi-targeted drugs which appeared to reduce drug resistance developing in cancer cells were favoured over single-

targeted drugs (Khan & Mukhtar, 2008; Talevi, 2015; Teiten, Eifes, Dicato, & Diederich, 2010; Zhu, Choi, & Shah, 2015). In our previous findings, it was revealed that DET from *E. scaber* suppressed cell growth via cell cycle arrest in HCT116 cells. However, the *in vitro* and *in vivo* anti-tumour underlying cell death signalling conferred by DET in colorectal cancer cells have yet to be discovered.

Apoptosis, a cellular self-destruction to maintain homeostasis and physiological processes, often exploited as one of the major approaches for the development of therapeutic agents against cancer. Mechanistically, apoptosis can be categorized into extrinsic and intrinsic pathways. Extrinsic pathway is executed by the ligation between ligands and death receptors leading to caspase cascades events and eventually apoptosis (Fulda & Debatin, 2006; Kroemer *et al.*, 2007; Scaffidi *et al.*, 1998). Caspases (cysteine-aspartic proteases) are proteolytic enzymes which are broadly known for their roles in apoptosis particularly as initiator caspases (caspase-10, caspase-8 and caspase-9) and executioner caspase-3 (McIlwain *et al.*, 2013). The activation of extrinsic pathway further recruits initiator caspase-8 or -10 and activates caspase-3 or intrinsic pathway via caspase-9 and caspase-3 (Fulda & Debatin, 2006; Fulda & Kogel, 2015; Kischkel *et al.*, 2001; Milhas *et al.*, 2005; Walczak & Krammer, 2000). In line with these findings, upon exposure of HCT116 cells to DET, DR5 and Fas receptors were activated and further led to recruitment of caspase-10 and caspase-8 which in turn activated caspase-3. Additionally, DET also activated caspase-9 and caspase-3 along with cleavage of PARP driven towards apoptosis in HCT116 cells. Thus, these data further implied the involvement of extrinsic pathway and intrinsic pathways in mediating apoptosis in DET-treated cells.

Multiple lines of evidence implied the important roles of pro-apoptotic and anti-apoptotic Bcl-2 proteins in governing the MOMP to induce apoptosis. An excess of pro-

apoptotic (Bax/Bak) over anti-apoptotic (Bcl-2/Bcl-xL) signals initiated MOMP resulting in depolarization of mitochondrial membrane potential (Lopez & Tait, 2015). Our study demonstrated that DET induced an increase of pro-apoptotic Bax and Bak proteins accompanied by downregulation of anti-apoptotic Bcl-2 and Bcl-xL proteins. This indicates oligomerization of Bax/Bak disrupted the mitochondrial membrane potential in executing apoptosis through inhibition of Bcl-2 and Bcl-xL proteins by DET. IAPs family members including XIAP and survivin due to its function as direct inhibitors of caspases to mitigate apoptosis is regarded as one of the target interests for cancer therapy (Deveraux *et al.*, 1999; Lopez & Tait, 2015). The release of apoptogenic molecules including Smac/DIABLO and Omi/Htr A2 attenuates IAPs generation to enhance apoptosis through successive activation of caspase cascades (Lopez & Tait, 2015). This is corroborated by our findings suggesting that treatment with DET abrogated survivin and XIAP concomitantly promoted the caspase cascades involving caspase-9 and -3 in HCT116 cells.

MAPK cascade transductions regulate a myriad of fundamental cellular events such as cell proliferation, autophagy and apoptosis under various environmental stress including oxidative stress. It has been well established that MAPK signalling is one of cardinal signalling pathways in eliciting cancer cell demise. Generally, JNK and p38 MAPKs are associated with the promotion of apoptosis whereas ERK1/2 plays an imperative role in cell proliferation (Frasch *et al.*, 1998; Herr *et al.*, 1999; Sanchez *et al.*, 1998). However, recent evidence has shown that overactivation of ERK1/2 favours apoptosis such as anticancer effect of taxol mediated apoptosis via ERK activation in MCF7 cells (Bacus *et al.*, 2001). Hispolon from fungus *Phellinus linteus* exerted potent apoptosis inducing and anti-tumour effect through activation of ERK1/2, p38 MAPK and JNK1/2 pathways (Hsieh *et al.*, 2014). Similarly, activation of p38 MAPK, ERK1/2 and JNK were detected in DET-treated cells. To assess the involvement of MAPK pathways in the DET-induced

apoptosis, inhibitors of ERK (UO126), JNK (SP600125) and p38 (SB202190) were utilized. Accordingly, the exposure to DET preceded by the treatment with UO126, SP600125 and SB202190 mitigated the cell growth inhibitory effect and apoptosis as evidenced by a decline in cleaved caspase-3 and cleaved PARP. These findings implied that activation of MAPK signalling pathway is closely associated with the DET-induced apoptosis in HCT116 cells.

A complex network of pathways regulates the survival and cell death modalities in cancer cells. Mounting evidence has highlighted the paramount importance of PI3K/Akt/mTOR signalling transduction in the malignant transformation and cell survival favouring the aberrant growth and drug resistance (Abrams *et al.*, 2010; Dai *et al.*, 2005; Hennessy *et al.*, 2005; Porta *et al.*, 2014). Phytochemicals were found to induce apoptosis cell death by targeting PI3K/Akt/mTOR pathway. For example, curcumin and neoalboconol activated cell death modality via inhibition of PI3K/Akt/mTOR pathway in EJ bladder cancer cells and nasopharyngeal cancer cells, respectively (Deng *et al.*, 2013; Wang, 2011). PDK1, the core stimulator of PI3K-mediated signalling, is phosphorylated to promote the phosphorylation of Akt at Thr308 (Alessi *et al.*, 1997). Our current study revealed that inhibition of cancer cell growth by DET was due to the attenuation of PI3K/Akt pathway, as substantiated by dephosphorylation of PDK1 concomitantly with inactivation of Akt phosphorylation (Thr308/Ser473). One of the major downstream mediators of PI3K/Akt signalling, mTOR, consists of two distinct multiprotein complexes which are mTORC1 and mTORC2 containing raptor and rictor, respectively (Guertin & Sabatini, 2007). The complete activation of Akt phorsphorylation at Ser473 is aided by mTORC2 followed by the activation of mTORC1 which promotes cellular survival and proliferation. It is conceivable that both mTORC1 and mTORC2 complexes are closely associated with phosphorylation of Akt (Sarbassov *et al.*, 2005). The present findings revealed that treatment with DET abolished Akt activation as a result of inhibition of

mTOR phosphorylation further hindering the growth of colorectal carcinoma cells. In addition, constitutive inactivation of Akt abrogated mTORC1 leading to inhibition of cell proliferation preceding the initiation of apoptosis and autophagy. Collectively, our results demonstrated that modulation of mTOR and Akt were correlated with the initiation of autophagy and apoptosis in DET-treated cells. Notably, PTEN, a negative regulator of PI3K/Akt pathway, frequently mutated in human colorectal cancer, directly suppressed the PI3K/Akt/mTOR signalling transduction to sensitize apoptosis via dephosphorylation of PIP3 to PIP2 (Molinari & Frattini, 2013). In consistent with this, DET-induced activation of PTEN led to attenuation of PI3K/Akt/mTOR signalling cascades which resulted in the decline of colorectal cancer cell survival.

Insulin-like growth factors (e.g. IGF-I and IGF-II), IGF-IR and IGF-binding proteins (IGFBPs) are involved in various pathogenesis including cancers. Binding of IGF-I to IGF1R further activates the survival pathways such as PI3K/Akt/mTOR which promotes colon tumourigenesis (Browne *et al.*, 2011; Terashima *et al.*, 2012). Therefore, targeting IGFR could restore and enhance the sensitivity towards chemotherapy which is a promising alternative for patients who develop drug resistance (Sanchez *et al.*, 2016). A tumour suppressor, IGFBP5, functions to attenuate cell proliferation by binding to IGF-I/IGF-II, to deter IGF-I/-II from inciting the activation of IGF1R that leads to mitigation of PI3K/Akt/mTOR pathway (Giovannucci, 2001; J. Wang *et al.*, 2015). Interestingly, our findings clearly suggested that DET-induced release of IGFBP5 resulted in reduction of IGF-I/-II that suppresses activation of IGF1sR, followed by growth inhibition via inactivation of PI3K/Akt pathway. The present findings are in agreement with the recent report on the inhibition of PI3K/Akt pathway through inactivation of IGF1R in gastric cancer (J. Chen *et al.*, 2016). Recent evidence has shown that PI3K/Akt pathway regulates numerous downstream signalling molecules including caspases, Bcl-2 family proteins and IAPs family proteins to transmit its anti-apoptotic signal (Osaki *et al.*, 2004). It is

conceivable that activation of Akt averts p-Bad from interacting with the anti-apoptotic Bcl-xL and Bcl-2 proteins or augments the release of IAPs including XIAP and survivin to inactivate the intrinsic pathway by antagonizing caspase-9 and caspase-3 (Dan *et al.*, 2004; Holcik *et al.*, 2001; Jin *et al.*, 2004). This is in accordance with our observations that DET suppressed the PI3K/Akt pathway leading to inhibition of survivin thereby facilitated apoptosis via caspase cascades in HCT116 cells.

Autophagy fell under heavy debate in recent years and was proposed to possess a double-edged sword effect either as a promoter or suppresser of tumour cells. In addition to apoptosis, there is growing interest in exploiting the tumour cellular fate determinant, autophagy, as a promising target for anticancer strategies (Amaravadi *et al.*, 2011). Interestingly, the present study has shown that DET induced autophagy which was substantiated by the increase in AO-stained acidic vesicular organelles (AVOs) and the formation of autophagosomes detected by the TEM analysis. Additionally, the drastic accumulation of LC3B-II proteins, an essential autophagy indicator, was observed upon exposure of HCT116 cells to DET which further suggested the induction of autophagy. It is well established that mTOR is a key negative effector of autophagy initiation (Kimmelman, 2011). Inhibition of mTOR triggers the onset of autophagy via vesicular elongation preceded by the amalgamation of Atg5, Atg12 and Atg7 which consequently led to the binding of phosphatidylethanolamine to LC3-I thus facilitating the lipidation of LC3-II (Fulda & Kogel, 2015; Geng & Klionsky, 2008). LC3 interacts with p62/SQSTM1 adaptor to allow autophagic degradation upon closure (Bjorkoy *et al.*, 2005; Ranjan & Srivastava, 2016). It has been reported that a number of natural-derived drugs such as rottlerin and betulinic acid act as autophagy and apoptosis inducers by modulating Atg proteins, LC3 and the degradation of p62 in prostate cancer stem cells and colon adenocarcinoma cells, respectively (Dutta *et al.*, 2016; Kumar *et al.*, 2014). In our study, the observations were in parallel with the notion supporting DET as an effective

autophagy inducer. Considerable studies have outlined the crosstalk between autophagy and apoptosis. The present findings revealed that Z-VAD-FMK induced the accumulation of LC3B-II indicating the attenuation of apoptosis. DET-treated cells drive autophagy induction. However, inhibition of autophagy reduced the cleavage of PARP and caspase-3 indicating that autophagy facilitates the initiation of apoptosis. Similarly, these findings corroborated with a report that celastrol could induce both apoptosis and autophagy whereby the suppression of apoptosis promotes autophagy while the withdrawal of autophagy induction reduces apoptosis (Li *et al.*, 2015).

ROS has been widely reported to exert detrimental role in regulating cell proliferation and tumourigenesis or beneficial role in mediating apoptosis and autophagy by damaging cellular components such as DNA, protein and lipids in cancer cells (Maiuri *et al.*, 2007). Mounting evidence has emphasized the role of ROS in the interference of multiple signalling pathways to provoke apoptotic signal by several anticancer agents, for instance, oxaliplatin, capsaicin and curcumin (Kim *et al.*, 2008; Su *et al.*, 2006; Zhang *et al.*, 2008). ROS generated by mitochondria leads to depolarization of mitochondrial membrane potential and induction of apoptosis. In accordance with these, our results demonstrated that DET instigated excessive release of ROS concomitantly with the occurrence of apoptosis induction in HCT116 cells. Intriguingly, the presence of ROS scavenger, NAC, reversed DET-induced MAPK activation concurrently with the deactivation of apoptosis and autophagy induction. In line with our findings, momordin lc was found to activate apoptotic and autophagic cell death via ROS/MAPK signalling cascades in human hepatocellular carcinoma cells (Mi *et al.*, 2016; Wang *et al.*, 2013). Therefore, it is conceivable that ROS functions as a prominent stimulator in the induction of apoptosis and autophagy in DET-treated cells.

To further verify the *in vitro* anticancer effect of DET, *in vivo* anti-tumour activity was evaluated. Our *in vivo* data demonstrated that the treatment with DET significantly inhibited the tumour growth along with the prominent enhancement in the survival of mice. In addition, it is clearly shown that no apparent signs of toxicity and weight loss was noted in the treated mice. These findings were corroborated with a previous toxicity findings which revealed the maximum tolerated dose for DET to be 40mg/kg in the mice model (Singh *et al.*, 2005). Disequilibrium between apoptosis and cell proliferation leads to colorectal tumourigenesis which is often linked to the proliferative determinant, PCNA. It has been well established that colorectal carcinoma exhibited high PCNA expression and thus is widely used as a biomarker to identify colorectal carcinoma (Guzinska *et al.*, 2009). PCNA is crucial for DNA replication during S-phase of cell cycle and hence accelerates DNA synthesis and enhances cell proliferation. Accordingly, our *in vivo* data showed that DET-induced blockade of tumour cell growth was substantiated by a significantly lower PCNA labelling index when compared to the control group. Furthermore, our immunohistochemical findings showed that DET down-modulated Bcl-2, activated Bax, cleaved caspase-3 and LC-II proteins which is in line with the *in vitro* results further indicating the potential of DET in targeting colorectal cancer via induction apoptosis and autophagy.

Based on our collective findings, the proposed mechanistic insight demonstrated the potent DET induced apoptosis and autophagy in *in vitro* and *in vivo* models (Figure 5.2). DET triggered extrinsic pathway by activation of death receptor subsequently led to intrinsic pathway via modulation of the Bcl-2 family member protein expression and caspase cascades activation resulting in cleavage of PARP, eventually apoptosis. Apart from this, anticancer effect of DET was shown to suppress cell growth attributed to the inactivation of PI3K/Akt/mTOR signalling involving inhibition of IGF-1sR by the IGFBP5 complexed with IGF-I. It is well documented that dephosphorylation of PTEN

further allows activation of PDK-1 leading to inactivation of Akt and the downstream signalling molecule, mTOR. Consequently, inactivation of PI3K/Akt/mTOR pathway first led to apoptosis by attenuating the production of IAPs and activating the cleavage of caspase-9 and -3. Alternatively, inactivation of PI3K/Akt/mTOR pathway resulted in conjugation of Atg12 and Atg 5 with the aid of Atg7. This led to the lipidation of LC3-I to LC3-II and facilitate the degradation of p62 eventually executed the autophagy. Additionally, JNK and p38 MAPK signalling were also found initiated by DET. Surprisingly, apoptosis via multiple signalling pathways including intrinsic, extrinsic and MAPK pathway conferred by DET, was potentiated by the oxidative stress stimuli, ROS. This study outlines a new insight on DET executing cell death via multiple signalling pathways thereby highlighting the potential of DET as a multi-functional therapeutic agent for colorectal cancer. Taken together, the *in vivo* evidences were in line with the *in vitro* observations demonstrating that DET induced apoptosis and autophagy in HCT116 cells. In summary, current findings are the first reported that DET from *E. scaber* possesses the *in vitro* and *in vivo* anti-tumour effect in HCT116 human colorectal carcinoma.

Great interest is emerging on utilization of natural-based anti-inflammatory agents to protect neuronal cell survival and target neuroinflammation which is commonly characterized by activated microglia in various neurodegenerative diseases (Block & Hong, 2005; Smith *et al.*, 2012). Based on the ethnomedicinal evidences, *E. scaber* was shown to possess manifold of health benefits which was traditionally used to treat eczema, arthritis, hepatitis and cancer. In Malaysia, Malay midwives traditionally used *E. scaber* as an oxytoxin aiding in the process of childbirth as well to counteract post-partum inflammation (Ho *et al.*, 2009). Several lines of scientific evidences supported the anti-inflammatory properties of *E. scaber* as demonstrated in different inflammatory agent-stimulated *in vitro* and *in vivo* models (Lin *et al.*, 1995; Rajesh & Latha, 2001).

Nevertheless, the anti-inflammatory effect of *E. scaber* and the underlying mechanisms in LPS-induced microglia cells remains elusive. Thus, our present findings were the first to reveal that ESEAF possessed anti-neuroinflammatory and antioxidative effects.

Aberrant release of inflammatory signalling molecules is primarily triggered upon the over activation of microglial cells which is manifested as a feature of neuroinflammation (Block *et al.*, 2007). It is well documented that excessive production of pro-inflammatory mediators such as NO and PGE2 by activated microglia is demonstrated to possess neurotoxic effects (Chao *et al.*, 1992; Jayasooriya *et al.*, 2015; Jeong *et al.*, 2015). Uncontrollable production of NO derived from L-arginine is attributed to the excessive iNOS generation which is known to be a potent neurotoxin in disintegrating DNA, lipids and proteins (Aktan, 2004; Bloodsworth *et al.*, 2000). Abnormal increase in COX-2, a rate-limiting enzyme, catalyses the synthesis of PGE2 which is strongly considered to be involved in inflammation-mediated cytotoxicity (Simon, 1999). It is conceivable that the concerted action of NO, iNOS, PGE2 and COX-2 resulted in relentless neuroinflammation which is evidently implicated in the pathogenesis of neurodegenerative disorders including AD (Block *et al.*, 2007; Minghetti, 2004). Notably, regulation of pro-inflammatory mediators in CNS is one of the potential approaches in targeting neuroinflammation. Accordingly, our results suggested that 2004). Notably, regulation of pro-inflammatory mediators in CNS is one of the potential approaches in targeting neuroinflammation. Accordingly, our results suggested that ESEAF prevented NO and PGE2 production in LPS-stimulated BV-2 cells pertaining to the transcriptional mitigation of COX-2 and iNOS suggesting its potential anti-neuroinflammatory effect. Transcription factor NF-κB functions as an integral molecule which orchestrates the onset of inflammatory response in the presence of oxidative stress stimulation (Mattson, 2001). Considerable evidence revealed that translocated NF-κB bound to the promoter region

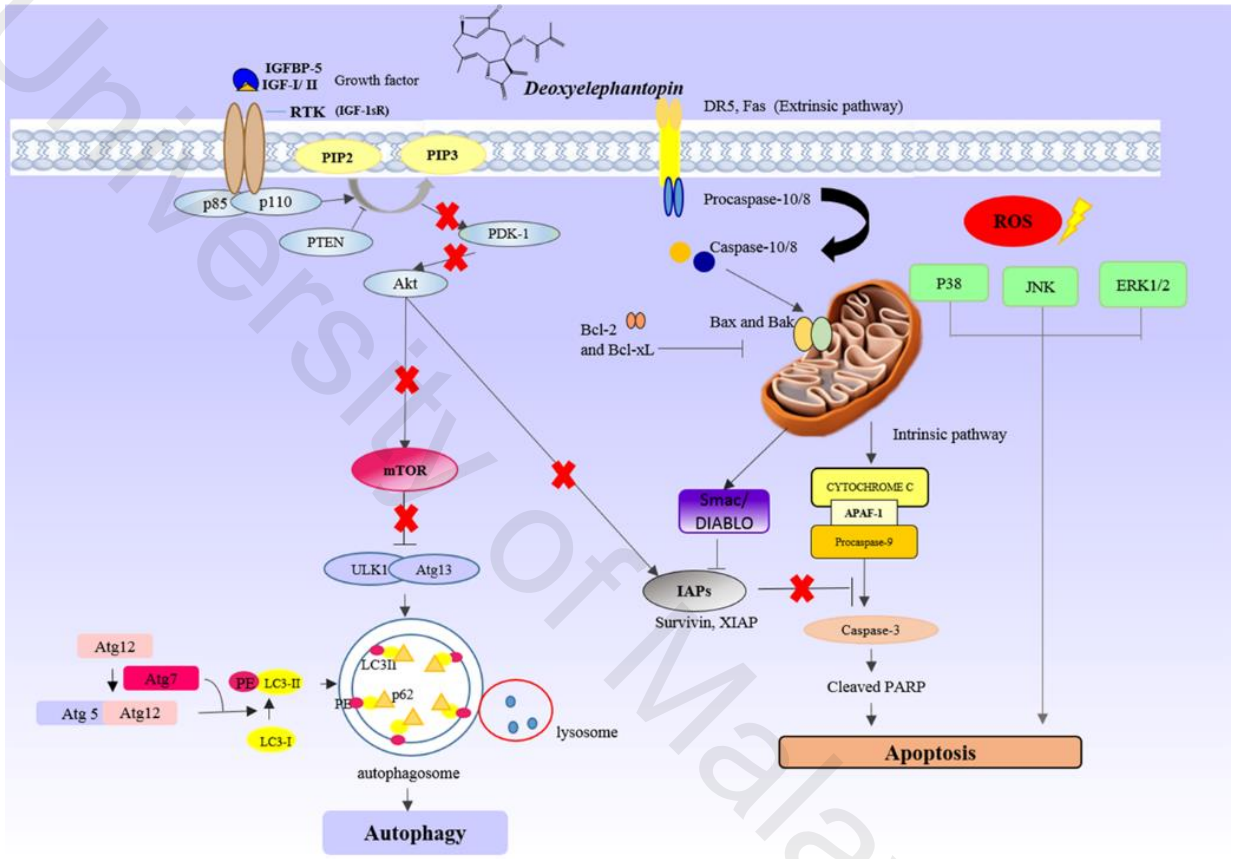


Figure 5.2: Schematic diagram demonstrated that multiple signalling pathways mediated by deoxyelephantopin in HCT116 colorectal cancer cells. DET mediated apoptosis involving caspase cascades and modulation of Bcl-2 family members in HCT116. Furthermore, DET, a ROS inducing agent, executed apoptosis via multiple signalling pathways including intrinsic, extrinsic and MAPK pathway via oxidative stress. Apart from this, cell growth inhibitory effect of DET was strengthened by the mitigation of PI3K/Akt signalling pathway which consequently led to induction of apoptosis and autophagy.

of pro-inflammatory genes encoding cytokines, COX-2 and iNOS which exacerbates inflammatory response and results in the development of neuroinflammatory-related diseases (Baeuerle & Baltimore, 1996; Karin *et al.*, 2004; Mattson, 2001). It has been proposed that dampening of NF-κB transcriptional activity may rescue the neurons from death and ameliorate the susceptibility towards neurodegenerative diseases which has become a promising target for the treatment of neuroinflammatory diseases. In addition, MAPK cascades encompassing p38, JNK and ERK are required in transcriptional changes of NF-κB during activation of microglia in response to several stimuli (Jung *et al.*, 2007; Kaminska, 2005). The p38 MAPK, also known as an important upstream signalling molecule, primarily activates NF-κB translocation followed by sequential stimulation of iNOS and COX-2 release in LPS-stimulated microglia (Kim *et al.*, 2006). This notion was supported by several lines of evidences in which the anti-neuroinflammatory agents, resveratrol and curcumenol, mitigated iNOS and COX-2 accompanied with the attenuation of NF-κB via the inactivation of p38 signalling (Lo *et al.*, 2015; Zhong *et al.*, 2012). In our present data, exposure to ESEAF prevented the p38 phosphorylation via blockade of NF-κB translocation which ultimately modulated LPS-stimulated iNOS and COX-2 in microglia cells, indicating ESEAF acts as a potent inhibitor of p38.

Constitutive activation of NF-κB is closely associated with the dysregulation of pro-inflammatory cytokines such as TNF-α and IL-1β upon sustained activation of microglia cells. The neurotoxic TNF-α and IL-1β cytokines may result in neuronal damage along with the accumulation of Aβ which exacerbates the development and progression of neurodegenerative diseases particularly AD (Chen *et al.*, 2012; Wang *et al.*, 2015). Hence, the modulation of pro-inflammatory cytokines, TNF-α and IL-1β, is one of potential strategies to mitigate neuroinflammation and thereby remove the deposition of Aβ in the brain (Heneka *et al.*, 2015). Considerable studies have revealed the anti-

neuroinflammatory agents such as celastrol and resveratrol suppressed the propagation of microglial via withdrawal of TNF-α and IL-1β (Jung *et al.*, 2007; Zhong *et al.*, 2012). Our present findings unveiled an interesting phenomenon that ESEAF hindered the inflammatory response by suppressing the production of pro-inflammatory TNF-α and IL-1β via the abrogation of LPS-induced NF-κB in BV-2 cells. This was further verified by the CAEE, a specific NF-κB inhibitor, which shared similar outcome as ESEAF-treated group. Thus, these findings suggested that the beneficial effects of ESEAF are attributable to the mitigation of neuroinflammatory responses.

Oxidative stress arises owing to the disequilibrium between generation of oxidants and antioxidant defence system creating a vicious cycle amplifying inflammation (Barnham *et al.*, 2004; Rosales *et al.*, 2010). In CNS, aberrant release of ROS promotes the activation of microglia which further provokes neuroinflammation responses resulting in progression of neurodegenerative disorders (Hsieh & Yang, 2013; Uttara *et al.*, 2009). Notably, aberrant production of ROS amplifies inflammatory responses which leads to release of pro-inflammatory components such as NO and PGE_2 in microglia via distinct signalling cascades such as NF-κB and MAPK (Park *et al.*, 2015; Rosenberger *et al.*, 2001). Numerous studies has suggested that combating oxidative stress in activated microglia exhibits as an anti-inflammatory therapeutic intervention for neuroinflammation-related diseases. This corroborated our findings in demonstrating that ESEAF restored redox balance by reversing LPS-induced ROS accumulation in BV-2 cells. These data suggested that ESEAF transforms the activated microglia back to quiescent state via detoxifying ROS.

Plants as a natural source of antioxidants has been proposed to function as free radical scavenger and possesses the ability in ameliorating the neuroinflammatory response (Block & Hong, 2005). In addition, antioxidants play a crucial role in the neutralization

of oxidative stress by terminating free radicals or scavenging ROS to prevent aberrant ROS production which could potently intensify the event of inflammation (Valko *et al.*, 2006). Hence, counteraction by antioxidants can attenuate neuroinflammation and the development of neurodegenerative diseases. Accordingly, ESEAF displayed a noteworthy DPPH scavenging activity implying that ESEAF contained a rich source of antioxidants. It is well established that excessive accumulation of neurotoxic factors such as NO, ROS and superoxide radicals (O_2^-) in the activated microglia is implicated in the pathogenesis of neurodegenerative diseases (Colton & Wilcock, 2010; Ha *et al.*, 2012). Therefore, enzymatic antioxidants including SOD, CAT and GPx act as crucial components in eliminating the detrimental free radicals to restore the redox balance in the cells (Limón & Gonsebatt, 2009). Superoxide radical (O_2^-) is catalysed by SOD enzymes yielding H_2O_2 and oxygen (Weydert & Cullen, 2010) while H_2O_2 is converted by catalase into water and oxygen. Based on the results obtained, present findings showed that ESEAF significantly enhanced catalase and superoxide anion scavenging activity which further validated the antioxidant potential of ESEAF.

Previous studies provided insights on the nuclear factor-2 erythroid related factor-2 (Nrf2) which is considered as an important regulator of the antioxidant response against oxidative insults (Hybertson *et al.*, 2011). Conversely, ablation of Nrf2 accompanied by the dysregulation of antioxidant defence system which renders cells more susceptible to the inflammatory response consequently leading to neuronal and brain injury. Hence, Nrf2 has recently raised great interest owing to their anti-neuroinflammatory properties. In this regard, ample evidence revealed that plant-derived therapeutic agents may activate antioxidant defence mechanism via Nrf2 cascades signalling against inflammation-related neurodegenerative diseases followed by abolishing oxidative stress via a series of antioxidant responsive genes encompassing HO-1, SOD and catalase (Gonzalez *et al.*, 2013; Lee *et al.*, 2013; Muiswinkel & Kuiperij, 2005). Our present findings are

corresponded to this notion indicating ESEAF promoted the translocation of Nrf2 to nucleus in LPS-stimulated microglia consequently enhance the antioxidant enzyme levels including SOD and catalase limiting the production of ROS. In addition, recent studies have shown that crosstalk between HO-1 and NF-κB is primarily orchestrated by Nrf2 which emerges as a potential therapeutic intervention to alleviate inflammation and microgliosis (Foresti *et al.*, 2013; Kang *et al.*, 2013; Paine *et al.*, 2010; Surh & Na, 2008). Mechanistically, oxidative stress stimulated Nrf2 to transcriptionally activate ARE encoding HO-1 gene followed by repression of NF-κB which led to a decline in inflammatory mediators such as iNOS and COX2 (Srisook & Cha, 2005; Wakabayashi *et al.*, 2010). Substantial evidences revealed that quercetin and 3,4,5-trihydroxycinnamic acid abrogated inflammatory response via Nrf2/HO-1 signalling involving NF-κB in BV-2 cells (Lee *et al.*, 2014; Sun *et al.*, 2015). This was corroborated with current data that enhancement of Nrf2 translocation along with induction of HO-1 further repressed NF-κB via modulation of the downstream pro-inflammatory molecules in BV-2 cells which stimulated by LPS. Present findings supported the potential of ESEAF in alleviating neuroinflammatory response. Collectively, the involvement of signalling pathways conferred by ESEAF in LPS-induced BV-2 cells was illustrated in Figure 5.3.

Since ancient times, herbal plant has always been traditionally used in treating human diseases (Leonti & Casu, 2013; Verma & Singh, 2008). However, some have been mistakenly regarded as safe due to the natural source and a lack of experimental evidence on the toxicity and adverse effect of these treatments. In the present study, the untreated group were administered with vehicle while the treated group was administered with ethyl acetate fraction. The toxic symptoms and mortality were observed daily for fourteen days in the mice. After 14 days treatment, mice which were orally dosed with the highest dose of 2000mg/kg showed absence of mortalities, distress or toxic symptoms. The body weight of the mice increased both in the control and treated groups. Other than body

weight, organ coefficient is an essential determinant of physiological and pathological status. Therefore, organ coefficient is essential to assess the damage to the organs including heart, kidney, liver, lungs and spleen triggered by the toxicant (Dybing *et al.*, 2002; Jothy *et al.*, 2011). In addition, the histopathological findings were corroborated with the organ and body weight evidenced by the negligible structural changes in the organs including heart, kidney, spleen, lungs and liver. This study reckoned that ESEAF may be considered as safe and possesses an LD_{50} value of greater than 2000mg/kg.

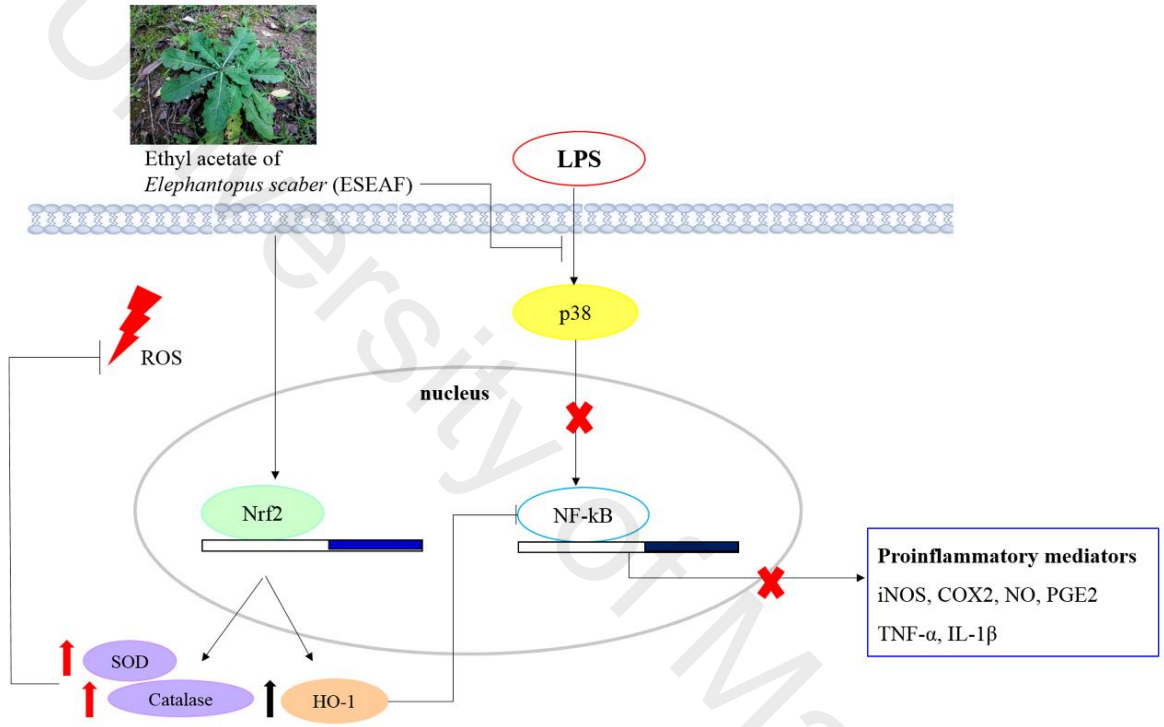


Figure 5.3: Schematic diagram illustrated the underlying anti-inflammatory pathways conferred by ESEAF on LPS-stimulated BV-2 microglia cells. Treatment with ESEAF attenuated LPS-stimulated NO, iNOS, COX-2, PGE_2, IL-1β and TNF-α through p38 and NF-κB inactivation. Apart from this, ESEAF, as a potent antioxidant, enhanced the antioxidant defence mechanism via Nrf2/HO-1signalling by scavenging oxidative stress and ameliorating the neuroinflammatory response.

CHAPTER 6: CONCLUSION

To the best of our knowledge, the present study is the first to provide new insight on the anticancer, anti-neuroinflammatory and antioxidant activities of *Elephantopus scaber*. Our current findings demonstrated that *E. scaber* possesses anticancer effect against HCT116 human colorectal cancer cells. The colorectal cancer cell growth inhibitory effect conferred by ESEAF via caspase-dependent mitochondrial-mediated apoptosis in response to oxidative stress stimulation. The anticancer potential of ESEAF was investigated further via bioassay-guided approach leading to the isolation of bioactive compound, deoxyelephantopin (DET). DET was shown to exert cytotoxic effect against HCT116 cells. The anti-proliferative effect of DET was observed in HCT116 cells whereby DET abrogated cell cycle progression via S and G2/M phase upon p53 and p21 activation. Cell cycle arrest triggered by DET was negatively coordinated by a concerted action of cyclins and CDKs encompassing CDK2, CDK4, cyclin B1, cyclin E2, cyclin D1 and cyclin A2. In addition, accumulation of apoptotic cell populations at sub G1 phase by DET was corroborated with the apoptosis inducing effects evidenced by the cleavage of caspase-3 and PARP. This led to further investigation on the apoptosis inducing effects underlying mechanisms of DET. Exposure to DET triggered apoptosis via intrinsic and extrinsic pathways involving modulation of Bcl-2 family members and activation of caspase cascades in HCT116 cells. One of the interesting findings revealed that the activation of MAPK signalling pathways is conceivable to be associated with the apoptotic response elicited by DET in HCT116 cells. Additionally, the cellular event of autophagy was activated by DET in response to ROS on HCT116 cells. Apart from this, growth inhibitory effect of DET was strongly correlated to the inactivation of PI3K/Akt signalling pathway which consequently resulted in the induction of apoptosis and autophagy. It is deduced that DET stimulated cell cycle arrest, apoptosis and autophagy via inhibition of PI3K/Akt/mTOR signalling and activation of ROS-dependent MAPK

pathway. More importantly, the *in vitro* study was validated by the *in vivo* data exhibiting a pronounced tumour growth suppression via apoptosis and autophagy. In summary, the compelling evidences outlined in this study shed new light on the enormous potential of DET as a multi-target therapeutic agent for colorectal cancer.

Our study also demonstrated that ESEAF exerts anti-neuroinflammatory activity against LPS-induced BV-2 cells. Exposure to ESEAF ameliorated inflammatory response by mitigating LPS-induced pro-inflammatory cytokines and molecules via inactivation of p38 and NF-κB in BV-2 cells. Furthermore, ESEAF also functions as a potent antioxidant to strengthen the antioxidant defense mechanism via Nrf2/HO-1 signalling to attenuate inflammatory response in microglia cells. Notably, ESEAF could be considered safe with no toxic symptoms observed (LD_{50}>2000mg/kg). The potential of ESEAF in targeting neuroinflammatory-related diseases such as Alzheimer's disease is attributed to the antioxidative and anti-inflammatory effects.

From past to present, *E. scaber* has been widely consumed as tea or decoction for preventive and curative purposes based on the manifold of ethnomedicinal benefits. Therefore, the ethnopharmacological claims of *E. scaber* related to anticancer and anti-inflammatory properties advocated by our collective evidence could potentially contribute to the pharmaceutical and nutraceutical industry for the intervention of cancer and neurodegenerative diseases.

REFERENCES

Abhimannue, A. P., Mohan, M. C., & B, P. K. (2016). Inhibition of tumor necrosis factor-alpha and interleukin-1beta production in lipopolysaccharide-stimulated monocytes by methanolic extract of *Elephantopus scaber* Linn and identification of bioactive components. *Applied Biochemistry & Biotechnology, 179*(3), 427-443.

Abrams, S. L., Steelman, L. S., Shelton, J. G., Wong, E. W., Chappell, W. H., Basecke, J., . . . McCubrey, J. A. (2010). The Raf/MEK/ERK pathway can govern drug resistance, apoptosis and sensitivity to targeted therapy. *Cell Cycle, 9*(9), 1781-1791.

Achuta, N., Shukla, S. S., & Rawat, A. K. S. (2010). An ethnobotanical study of medicinal plants of Rewa district Madhya Pradesh. *Indian Journal of Traditional Knowledge, 9*(1), 191-202.

Adams, J. M. (2003). Ways of dying: multiple pathways to apoptosis. *Genes & Development, 17*(20), 2481-2495.

Ahmad, N., Cheng, P., & Mukhtar, H. (2000). Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochemical and Biophysical Research Communications, 275*(2), 328-334.

Ahmed, S. M., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2017). Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica et Biophysica Acta, 1863*(2), 585-597.

Akaneya, Y., Takahashi, M., & Hatanaka, H. (1995). Interleukin-1 beta enhances survival and interleukin-6 protects against MPP+ neurotoxicity in cultures of fetal rat dopaminergic neurons. *Experimental Neurology, 136*(1), 44-52.

Aktan, F. (2004). iNOS-mediated nitric oxide production and its regulation. *Life Sciences, 75*(6), 639-653.

Alderton, W. K., Cooper, C. E., & Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal, 357*(Pt 3), 593-615.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current Biology, 7*(4), 261-269.

Allan, S. M., Tyrrell, P. J., & Rothwell, N. J. (2005). Interleukin-1 and neuronal injury. *Nature Review Immunology, 5*(8), 629-640.

Amaravadi, R. K., Lippincott-Schwartz, J., Yin, X. M., Weiss, W. A., Takebe, N., Timmer, W., . . . White, E. (2011). Principles and current strategies for targeting autophagy for cancer treatment. *Clinical Cancer Research, 17*(4), 654-666.

Amor, S., Puentes, F., Baker, D., & van der Valk, P. (2010). Inflammation in neurodegenerative diseases. *Immunology, 129*(2), 154-169.

Amorim, M. H., Gil da Costa, R. M., Lopes, C., & Bastos, M. M. (2013). Sesquiterpene lactones: adverse health effects and toxicity mechanisms. *Critical Reviews in Toxicology, 43*(7), 559-579.

Andersen, J. K. (2004). Oxidative stress in neurodegeneration: cause or consequence? *Nature Medicine, 10 Suppl*, S18-25.

Angelopoulos, P., Agouridaki, H., Vaiopoulos, H., Siskou, E., Doutsou, K., Costa, V., & Baloyiannis, S. I. (2008). Cytokines in Alzheimer's disease and vascular dementia. *International Journal of Neuroscience, 118*(12), 1659-1672.

Anitha, V. T., Antonisamy, J. M., & Jeeva, S. (2012). Anti-bacterial studies on Hemigraphis colorata (Blume) H.G. Hallier and *Elephantopus scaber* L. *Asian Pacific Journal of Tropical Medicine, 5*(1), 52-57.

Arcaro, A., & Guerreiro, A. S. (2007). The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications. *Current Genomics, 8*(5), 271-306.

Asghar, U., Witkiewicz, A. K., Turner, N. C., & Knudsen, E. S. (2015). The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nature reviews Drug discovery, 14*(2), 130-146.

Ashkenazi, A. (2008a). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Review, 19*(3-4), 325-331.

Ashkenazi, A. (2008b). Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nature Reviews Drug Discovery, 7*(12), 1001-1012.

Aslam, M. S., Ahmad, M. S., Mamat, A. S., Ahmad, M. Z., & Salam, F. (2016). Antioxidant and Wound Healing Activity of Polyherbal Fractions of *Clinacanthus nutans* and *Elephantopus scaber*. *Evidence Based Complementary Alternative Medicine, 2016*, 4685246.

Azevedo, F. A., Carvalho, L. R., Grinberg, L. T., Farfel, J. M., Ferretti, R. E., Leite, R. E., . . . Herculano-Houzel, S. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *Journal of Comparative Neurology, 513*(5), 532-541.

Bacus, S. S., Gudkov, A. V., Lowe, M., Lyass, L., Yung, Y., Komarov, A. P., . . . Seger, R. (2001). Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. *Oncogene, 20*(2), 147-155.

Baeuerle, P. A. (1998). IkappaB-NF-kappaB structures: at the interface of inflammation control. *Cell, 95*(6), 729-731.

Baeuerle, P. A., & Baltimore, D. (1996). NF-kappa B: ten years after. *Cell, 87*(1), 13-20.

Baeuerle, P. A., & Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. *Annual Review Immunology, 12,* 141-179.

Bai, L., & Wang, S. (2014). Targeting apoptosis pathways for new cancer therapeutics. *Annual Review Medicine, 65,* 139-155.

Baig, S., Seevasant, I., Mohamad, J., Mukheem, A., Huri, H. Z., & Kamarul, T. (2016). Potential of apoptotic pathway-targeted cancer therapeutic research: Where do we stand? *Cell Death & Disease, 7,* e2058.

Balachandran, P., & Govindarajan, R. (2005). Cancer--an ayurvedic perspective. *Pharmacological Research, 51*(1), 19-30.

Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., . . . Vercellotti, G. M. (1992). Ferritin: a cytoprotective antioxidant strategem of endothelium. *Journal of Biological Chemistry, 267*(25), 18148-18153.

Barnham, K. J., Masters, C. L., & Bush, A. I. (2004). Neurodegenerative diseases and oxidative stress. *Nature reviews Drug discovery, 3*(3), 205-214.

Bartek, J., Bartkova, J., & Lukas, J. (2007). DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene, 26*(56), 7773-7779.

Bassermann, F., Eichner, R., & Pagano, M. (2014). The ubiquitin proteasome system - implications for cell cycle control and the targeted treatment of cancer. *Biochimica et Biophysica Acta, 1843*(1), 150-162.

Becher, B., Spath, S., & Goverman, J. (2017). Cytokine networks in neuroinflammation. *Nature Review Immunology, 17*(1), 49-59.

Becker, C., Fantini, M. C., Schramm, C., Lehr, H. A., Wirtz, S., Nikolaev, A., . . . Neurath, M. F. (2004). TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity, 21*(4), 491-501.

Behera, S. K., and Misra, M. K. (2005). Indigenous phytotherapy for genito-urinary diseases used by the Kandha tribe of Orissa, India. *Journal of Ethnopharmacology, 102*(3), 319-325.

Bieging, K. T., Mello, S. S., & Attardi, L. D. (2014). Unravelling mechanisms of p53-mediated tumour suppression. *Nature Reviews Cancer, 14*(5), 359-370.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal, 5*(1), 9-19.

Birch, A. M., Katsouri, L., & Sastre, M. (2014). Modulation of inflammation in transgenic models of Alzheimer's disease. *J Neuroinflammation, 11,* 25.

Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., . . . Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *Journal of Cell Biology, 171*(4), 603-614.

Block, M. L., & Hong, J.-S. (2005). Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Progress in Neurobiology, 76*(2), 77-98.

Block, M. L., & Hong, J. S. (2005). Microglia and inflammation-mediated neurodegeneration: Multiple triggers with a common mechanism. *Progress in Neurobiology, 76*(2), 77-98.

Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nature Reviews Neuroscience, 8*(1), 57-69.

Bloodsworth, A., O'Donnell, V. B., & Freeman, B. A. (2000). Nitric oxide regulation of free radical- and enzyme-mediated lipid and lipoprotein oxidation. *Arteriosclerosis Thrombosis and Vascular Biolology, 20*(7), 1707-1715.

Borner, C. (2003). The Bcl-2 protein family: Sensors and checkpoints for life-or-death decisions. *Molecular Immunology, 39*(11), 615-647.

Bratton, S. B., & Salvesen, G. S. (2010). Regulation of the Apaf-1-caspase-9 apoptosome. *Journal of Cell Science, 123*(Pt 19), 3209-3214.

Breder, C. D., Tsujimoto, M., Terano, Y., Scott, D. W., & Saper, C. B. (1993). Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system. *Journal of Comparative Neurology, 337*(4), 543-567.

Bressenot, A., Marchal, S., Bezdetnaya, L., Garrier, J., Guillemin, F., & Plenat, F. (2009). Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. *Journal of Histochemistry Cytochemistry, 57*(4), 289-300.

Broker, L. E., Kruyt, F. A., & Giaccone, G. (2005). Cell death independent of caspases: A review. *Clinical Cancer Research, 11*(9), 3155-3162.

Brown, M. A., & Hural, J. (1997). Functions of IL-4 and control of its expression. *Critical Review Immunology, 17*(1), 1-32.

Browne, B. C., Crown, J., Venkatesan, N., Duffy, M. J., Clynes, M., Slamon, D., & O'Donovan, N. (2011). Inhibition of IGF1R activity enhances response to trastuzumab in HER-2-positive breast cancer cells. *Annals of Oncology, 22*(1), 68-73.

Buendia, I., Michalska, P., Navarro, E., Gameiro, I., Egea, J., & Leon, R. (2016). Nrf2-ARE pathway: An emerging target against oxidative stress and neuroinflammation in neurodegenerative diseases. *Pharmacology & Therapeutics, 157*, 84-104.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., . . . Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science, 282*(5393), 1497-1501.

But, P. P. H., Hon, P. M., Cao, H., Chan, D. T. W., Wu, B. M., Mak, T. C. W., & Che, C. T. (1997). Sesquiterpene lactones from *Elephantopus scaber*. *Phytochemistry, 44*(1), 113-116.

Butterfield, D. A., Castegna, A., Lauderback, C. M., & Drake, J. (2002). Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiology of Aging, 23*(5), 655-664.

Campbell, I. L., Abraham, C. R., Masliah, E., Kemper, P., Inglis, J. D., Oldstone, M. B., & Mucke, L. (1993). Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proceedings of National Academy of Sciences United States of America, 90*(21), 10061-10065.

Canales, M., Hernandez, T., Caballero, J., Romo de Vivar, A., Avila, G., Duran, A., & Lira, R. (2005). Informant consensus factor and antibacterial activity of the medicinal plants used by the people of San Rafael Coxcatlan, Puebla, Mexico. *Journal of Ethnopharmacology, 97*(3), 429-439.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science, 296*(5573), 1655-1657.

Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiology and Molecular Biology Reviews, 75*(1), 50-83.

Carracedo, A., & Pandolfi, P. P. (2008). The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene, 27*(41), 5527-5541.

Carson, M. J., Doose, J. M., Melchior, B., Schmid, C. D., & Ploix, C. C. (2006). CNS immune privilege: hiding in plain sight. *Immunological Reviews, 213*, 48-65.

Chaabane, W., User, S. D., El-Gazzah, M., Jaksik, R., Sajjadi, E., Rzeszowska-Wolny, J., & Los, M. J. (2013). Autophagy, apoptosis, mitoptosis and necrosis: Interdependence between those pathways and effects on cancer. *Archivum Immunologiae et Therapiae Experimentalis, 61*(1), 43-58.

Chadwick, M., Trewin, H., Gawthrop, F., & Wagstaff, C. (2013). Sesquiterpenoids lactones: Benefits to plants and people. *International Journal of Molecular Sciences, 14*(6), 12780-12805.

Chan, C. K., Supriady, H., Goh, B. H., & Kadir, H. A. (2015). *Elephantopus scaber* induces apoptosis through ROS-dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. *Journal of Ethnopharmacology, 168*, 291-304.

Chan, P. H. (2001). Reactive oxygen radicals in signaling and damage in the ischemic brain. *Journal of Cerebral Blood Flow Metabolism, 21*(1), 2-14.

Chang, C. L., Shen, C. C., Ni, C. L., & Chen, C. C. (2011). A new sesquiterpene from *Elephantopus scaber*. *HungKuang Journal, 65*, 49-56.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature, 410*(6824), 37-40.

Chao, C. C., Hu, S., Molitor, T. W., Shaskan, E. G., & Peterson, P. K. (1992). Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *Journal of Immunology, 149*(8), 2736-2741.

Chen, C. H., Zhou, W., Liu, S., Deng, Y., Cai, F., Tone, M., . . . Song, W. (2012). Increased NF-kappaB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *International Journal of Neuropsychopharmacology, 15*(1), 77-90.

Chen, F. E., Huang, D. B., Chen, Y. Q., & Ghosh, G. (1998). Crystal structure of p50/p65 heterodimer of transcription factor NF-kappaB bound to DNA. *Nature, 391*(6665), 410-413.

Chen, J., Guo, J., Chen, Z., Wang, J., Liu, M., & Pang, X. (2016). Linifanib (ABT-869) potentiates the efficacy of chemotherapeutic agents through the suppression of receptor tyrosine kinase-mediated AKT/mTOR signaling pathways in gastric cancer. *Scientific Reports, 6*, 29382.

Chen, J. C., Hsieh, M. J., Chen, C. J., Lin, J. T., Lo, Y. S., Chuang, Y. C., . . . Chen, M. K. (2016). Polyphyllin G induce apoptosis and autophagy in human nasopharyngeal cancer cells by modulation of AKT and mitogen-activated protein kinase pathways *in vitro* and *in vivo*. *Oncotarget, 7*(43), 70276-70289.

Chen, L. F., & Greene, W. C. (2004). Shaping the nuclear action of NF-kappaB. *Nature Reviews Molecular Cell Biology, 5*(5), 392-401.

Chen, W. W., Zhang, X., & Huang, W. J. (2016). Role of neuroinflammation in neurodegenerative diseases (Review). *Molecular Medicine Reports, 13*(4), 3391-3396.

Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., . . . Cobb, M. H. (2001). MAP kinases. *Chemical Reviews, 101*(8), 2449-2476.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., & Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science, 303*(5660), 1010-1014.

Choi, S. J., Lee, K. H., Park, H. S., Kim, S. K., Koh, C. M., & Park, J. Y. (2005). Differential expression, shedding, cytokine regulation and function of TNFR1 and TNFR2 in human fetal astrocytes. *Yonsei Medical Journal, 46*(6), 818-826.

Colton, C., & Wilcock, D. M. (2010). Assessing activation states in microglia. *CNS Neurological Disorders Drug Targets, 9*(2), 174-191.

Cragg, G. M., & Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100*(1-2), 72-79.

Cragg, G. M., & Newman, D. J. (2013). Natural products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta, 1830*(6), 3670-3695.

Craggs, L., & Kalaria, R. N. (2011). Revisiting dietary antioxidants, neurodegeneration and dementia. *Neuroreport, 22*(1), 1-3.

Cressman, D. E., Greenbaum, L. E., DeAngelis, R. A., Ciliberto, G., Furth, E. E., Poli, V., & Taub, R. (1996). Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science, 274*(5291), 1379-1383.

Cui, Q., Yu, J. H., Wu, J. N., Tashiro, S., Onodera, S., Minami, M., & Ikejima, T. (2007). P53-mediated cell cycle arrest and apoptosis through a caspase-3- independent, but caspase-9-dependent pathway in oridonin-treated MCF-7 human breast cancer cells. *Acta Pharmacologica Sinica, 28*(7), 1057-1066.

Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W., & Diehl, J. A. (2004). The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: Oxidative stress sensing by a Cul3-Keap1 ligase. *Molecular and Cellular Biology, 24*(19), 8477-8486.

Cunningham, J. M., Christensen, E. R., Tester, D. J., Kim, C. Y., Roche, P. C., Burgart, L. J., & Thibodeau, S. N. (1998). Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Research, 58*(15), 3455-3460.

Dai, D. L., Martinka, M., and Li, G. (2005). Prognostic significance of activated Akt expression in melanoma: a clinicopathologic study of 292 cases. *Journal of Clinical Oncology, 23*(7), 1473-1482.

Daisy, P., Jasmine, R., Ignacimuthu, S., & Murugan, E. (2009). A novel steroid from *Elephantopus scaber* L. an ethnomedicinal plant with antidiabetic activity. *Phytomedicine, 16*(2-3), 252-257.

Dan, H. C., Sun, M., Kaneko, S., Feldman, R. I., Nicosia, S. V., Wang, H. G., . . . Cheng, J. Q. (2004). Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *Journal of Biological Chemistry, 279*(7), 5405-5412.

Dan, I., Watanabe, N. M., & Kusumi, A. (2001). The Ste20 group kinases as regulators of MAP kinase cascades. *Trends in Cell Biology, 11*(5), 220-230.

Danielsen, S. A., Eide, P. W., Nesbakken, A., Guren, T., Leithe, E., & Lothe, R. A. (2015). Portrait of the PI3K/AKT pathway in colorectal cancer. *Biochimica et Biophysica Acta, 1855*(1), 104-121.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell, 103*(2), 239-252.

Dawson, V. L., & Dawson, T. M. (1998). Nitric oxide in neurodegeneration. *Progress Brain Research, 118*, 215-229.

Day, B. J. (2009). Catalase and glutathione peroxidase mimics. *Biochemical Pharmacology, 77*(3), 285-296.

de Silva, L. B., Herath, W. H. M. W., Jennings, R. C., Mahendrant, M., & Wannigama, G.E. (1982). A new sesquiterpene lactone from *Elephantopus scaber*. *Phytochemistry, 21*(5), 1173-1175.

Dean, R. T., Roberts, C. R., & Jessup, W. (1985). Fragmentation of extracellular and intracellular polypeptides by free radicals. *Progress in Clinical and Biological Research, 180*, 341-350.

Degterev, A., Boyce, M., & Yuan, J. (2003). A decade of caspases. *Oncogene, 22*(53), 8543-8567.

Deng, Q., Yu, X., Xiao, L., Hu, Z., Luo, X., Tao, Y., . . . Cao, Y. (2013). Neoalbaconol induces energy depletion and multiple cell death in cancer cells by targeting PDK1-PI3-K/Akt signaling pathway. *Cell Death and Disease, 4*, e804.

Deveraux, Q. L., Leo, E., Stennicke, H. R., Welsh, K., Salvesen, G. S., & Reed, J. C. (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO Journal, 18*(19), 5242-5251.

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene, 26*(22), 3279-3290.

Dinarello, C. A. (1996). Biologic basis for interleukin-1 in disease. *Blood, 87*(6), 2095-2147.

Dominguez-Brauer, C., Thu, K. L., Mason, J. M., Blaser, H., Bray, M. R., & Mak, T. W. (2015). Targeting mitosis in cancer: Emerging strategies. *Molecular Cell, 60*(4), 524-536.

Dotto, G. P. (2000). p21(WAF1/Cip1): More than a break to the cell cycle? *Biochimica et Biophysica Acta, 1471*(1), M43-56.

Duh, E. J., Maury, W. J., Folks, T. M., Fauci, A. S., & Rabson, A. B. (1989). Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proceedings of National Academy of Sciences United States of America, 86*(15), 5974-5978.

Duriez, P. J., & Shah, G. M. (1997). Cleavage of poly(ADP-ribose) polymerase: A sensitive parameter to study cell death. *Biochemistry and Cell Biology, 75*(4), 337-349.

Dutta, D., Chakraborty, B., Sarkar, A., Chowdhury, C., & Das, P. (2016). A potent betulinic acid analogue ascertains an antagonistic mechanism between autophagy and proteasomal degradation pathway in HT-29 cells. *BMC Cancer, 16*, 23.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'brien, J., Renwick, A., . . . Walker, R. (2002). Hazard characterisation of chemicals in food and diet: Dose response, mechanisms and extrapolation issues. *Food and Chemical Toxicology, 40*(2), 237-282.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., . . . Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell, 75*(4), 817-825.

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology, 35*(4), 495-516.

Engelman, J. A., Luo, J., & Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nature Reviews Genetics, 7*(8), 606-619.

Faivre, S., Kroemer, G., & Raymond, E. (2006). Current development of mTOR inhibitors as anticancer agents. *Nature Reviews Drug Discovery, 5*(8), 671-688.

Falasca, M., & Maffucci, T. (2012). Regulation and cellular functions of class II phosphoinositide 3-kinases. *Biochemical Journal, 443*(3), 587-601.

Fan, T. J., Han, L. H., Cong, R. S., & Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica et Biophysica Sinica (Shanghai), 37*(11), 719-727.

Farha, A. K., Dhanya, S. R., Mangalam, S. N., Geetha, B. S., Latha, P. G., & Remani, P. (2014). Deoxyelephantopin impairs growth of cervical carcinoma SiHa cells and induces apoptosis by targeting multiple molecular signaling pathways. *Cell Biology and Toxicology, 30*(6), 331-343.

Farha, A. K., Dhanya, S. R., Mangalam, S. N., & Remani, P. (2015). Anti-metastatic effect of deoxyelephantopin from *Elephantopus scaber* in A549 lung cancer cells *in vitro*. *Natural Products Research, 29*(24), 2341-2345.

Farnsworth, N. R., Akerele, O., Bingel, A. S., Soejarto, D. D., & Guo, Z. (1985). Medicinal plants in therapy. *Bull World Health Organ, 63*(6), 965-981.

Fattori, E., Lazzaro, D., Musiani, P., Modesti, A., Alonzi, T., & Ciliberto, G. (1995). IL-6 expression in neurons of transgenic mice causes reactive astrocytosis and increase in ramified microglial cells but no neuronal damage. *European Journal of Neuroscience, 7*(12), 2441-2449.

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell, 61*(5), 759-767.

Ferrandiz, M. L., & Devesa, I. (2008). Inducers of heme oxygenase-1. *Current Pharmaceutical Design, 14*(5), 473-486.

Ferris, C. D., Jaffrey, S. R., Sawa, A., Takahashi, M., Brady, S. D., Barrow, R. K., . . . Snyder, S. H. (1999). Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nature Cell Biology, 1*(3), 152-157.

Fischer, M. (2017). Census and evaluation of p53 target genes. *Oncogene, 36*(28), 3943-3956.

Fischer, M., Quaas, M., Steiner, L., & Engeland, K. (2016). The p53-p21-DREAM-CDE/CHR pathway regulates G2/M cell cycle genes. *Nucleic Acids Research, 44*(1), 164-174.

Fischer, R., & Maier, O. (2015). Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. *Oxidative Medicine Cellular Longevity, 2015*, 610813.

Foresti, R., Bains, S. K., Pitchumony, T. S., de Castro Brás, L. E., Drago, F., Dubois-Randé, J.-L., . . . Motterlini, R. (2013). Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells. *Pharmacological Research, 76*, 132-148.

Fox, J. L., & MacFarlane, M. (2016). Targeting cell death signalling in cancer: Minimising 'Collateral damage'. *British Journal of Cancer, 115*(1), 5-11.

Frasch, S. C., Nick, J. A., Fadok, V. A., Bratton, D. L., Worthen, G. S., & Henson, P. M. (1998). p38 mitogen-activated protein kinase-dependent and -independent intracellular signal transduction pathways leading to apoptosis in human neutrophils. *Journal of Biological Chemistry, 273*(14), 8389-8397.

Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T., & Yoshimori, T. (2008). The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Molecular Biology Cell, 19*(5), 2092-2100.

Fulda, S. (2015). Targeting extrinsic apoptosis in cancer: Challenges and opportunities. *Seminars Cell and Development Biology, 39*, 20-25.

Fulda, S., & Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene, 25*(34), 4798-4811.

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: Cell survival and cell death. *International Journal of Cell Biology, 2010*, 214074.

Fulda, S., & Kogel, D. (2015). Cell death by autophagy: Emerging molecular mechanisms and implications for cancer therapy. *Oncogene, 34*(40), 5105-5113.

Fulda, S., & Vucic, D. (2012). Targeting IAP proteins for therapeutic intervention in cancer. *Nature Reviews Drug Discovery, 11*(2), 109-124.

Gabrielli, B., Brooks, K., and Pavey, S. (2012). Defective cell cycle checkpoints as targets for anti-cancer therapies. *Frontiers in Pharmacology, 3*, 9.

Gach, K., Dlugosz, A., & Janecka, A. (2015). The role of oxidative stress in anticancer activity of sesquiterpene lactones. *Naunyn Schmiedebergs Archives Pharmacology, 388*(5), 477-486.

Galadari, S., Rahman, A., Pallichankandy, S., & Thayyullathil, F. (2017). Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radical Biology and Medicine, 104*, 144-164.

Galluzzi, L., Maiuri, M. C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., &Kroemer, G. (2007). Cell death modalities: classification and pathophysiological implications. *Cell Death and Differentiation, 14*(7), 1237-1243.

Gavet, O., & Pines, J. (2010). Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Developmental Cell, 18*(4), 533-543.

Geetha, B. S., Latha, P. G., &Remani, P. (2010). Evaluation of *Elephantopus scaber* on the inhibition of chemical carcinogenesis and tumor development in mice. *Pharmaceutical Biology, 48*(3), 342-348.

Geiersbach, K. B., & Samowitz, W. S. (2011). Microsatellite instability and colorectal cancer. *Archives of Pathology & Laboratory Medicine, 135*(10), 1269-1277.

Genestra, M. (2007). Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular Signalling, 19*(9), 1807-1819.

Geng, J., & Klionsky, D. J. (2008). The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO Reports, 9*(9), 859-864.

Giordano, O. S., Guerreiro, E., Pestchanker, M. J., Guzman, J., Pastor, D., & Guardia, T. (1990). The gastric cytoprotective effect of several sesquiterpene lactones. *Journal of Natural Products, 53*(4), 803-809.

Giovannucci, E. (2001). Insulin, insulin-like growth factors and colon cancer: A review of the evidence. *Journal of Nutrition, 131*(11 Suppl), 3109S-3120S.

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., & Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell, 140*(6), 918-934.

Gonzalez, R. S., Guzman, B. S., Medina, C. O. N., & Pedraza, C. J. (2013). Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. *Oxidative Medicine Cellular Longevity, 2013*, 801418.

Gorrini, C., Harris, I. S., & Mak, T. W. (2013). Modulation of oxidative stress as an anticancer strategy. *Nature Reviews Drug Discovery, 12*(12), 931-947.

Gottifredi, V., McKinney, K., Poyurovsky, M. V., & Prives, C. (2004). Decreased p21 levels are required for efficient restart of DNA synthesis after S phase block. *Journal of Biological Chemistry, 279*(7), 5802-5810.

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629.

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G. Y., Vallabhapurapu, S., . . . Karin, M. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell, 15*(2), 103-113.

Guertin, D. A., & Sabatini, D. M. (2007). Defining the role of mTOR in cancer. *Cancer Cell, 12*(1), 9-22.

Gupta, A. K., Cerniglia, G. J., Mick, R., Ahmed, M. S., Bakanauskas, V. J., Muschel, R. J., & McKenna, W. G. (2003). Radiation sensitization of human cancer cells *in vivo* by inhibiting the activity of PI3K using LY294002. *International Journal of Radiation Oncology Biology Physics, 56*(3), 846-853.

Gupta, S. C., Kim, J. H., Prasad, S., & Aggarwal, B. B. (2010). Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer and Metastasis Reviews, 29*(3), 405-434.

Gurib-Fakim, A., Sewraj, M., Gueho, J., & Dulloo, E. (1993). Medicalethnobotany of some weeds of Mauritius and Rodrigues. *Journal of Ethnopharmacology, 39*(3), 175-185.

Guzinska-Ustymowicz, K., Pryczynicz, A., Kemona, A., & Czyzewska, J. (2009). Correlation between proliferation markers: PCNA, Ki-67, MCM-2 and antiapoptotic protein Bcl-2 in colorectal cancer. *Anticancer Research, 29*(8), 3049-3052.

Ha, S. K., Moon, E., Lee, P., Ryu, J. H., Oh, M. S., & Kim, S. Y. (2012). Acacetin attenuates neuroinflammation via regulation the response to LPS stimuli *in vitro* and *in vivo*. *Neurochemistry Research, 37*(7), 1560-1567.

Halliwell, B., & Gutteridge, J.M.C. (2007). Free Radicals in Biology and Medicine (Vol. fourth edition): Oxford University Press.

Hama, T., Kushima, Y., Miyamoto, M., Kubota, M., Takei, N., & Hatanaka, H. (1991). Interleukin-6 improves the survival of mesencephalic catecholaminergic and septal cholinergic neurons from postnatal, two-week-old rats in cultures. *Neuroscience, 40*(2), 445-452.

Hammer, M. L., & Johns, E. A. (1993). Tapping an Amazonian plethora: four medicinal plants of Marajo Island, Para (Brazil). *Journal of Ethnopharmacology, 40*(1), 53-75.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hanisch, U. K., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature Neuroscience, 10*(11), 1387-1394.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., & Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell, 75*(4), 805-816.

Harris, I. S., & Brugge, J. S. (2015). Cancer: The enemy of my enemy is my friend. *Nature, 527*(7577), 170-171.

Hartwell, L. H., & Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science, 246*(4930), 629-634.

Hasima, N., & Ozpolat, B. (2014). Regulation of autophagy by polyphenolic compounds as a potential therapeutic strategy for cancer. *Cell Death and Disease, 5*, e1509.

Hay, N., & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes & Development, 18*(16), 1926-1945.

Hayden, M. S., & Ghosh, S. (2004). Signaling to NF-kappaB. *Genes & Development, 18*(18), 2195-2224.

Hayden, M. S., & Ghosh, S. (2008). Shared principles in NF-kappaB signaling. *Cell, 132*(3), 344-362.

Hayes, J. D., McMahon, M., Chowdhry, S., & Dinkova-Kostova, A. T. (2010). Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. *Antioxidant & Redox Signaling, 13*(11), 1713-1748.

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., . . . Kummer, M. P. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurology, 14*(4), 388-405.

Heneka, M. T., Kummer, M. P., & Latz, E. (2014). Innate immune activation in neurodegenerative disease. *Nature Review Immunology, 14*(7), 463-477.

Hennessy, B. T., Smith, D. L., Ram, P. T., Lu, Y., &Mills, G. B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nature Reviews Drug Discovery, 4*(12), 988-1004.

Herr, I., Wilhelm, D., Meyer, E., Jeremias, I., Angel, P., & Debatin, K. M. (1999). JNK/SAPK activity contributes to TRAIL-induced apoptosis. *Cell Death and Differentiation, 6*(2), 130-135.

Hiradeve, S. M., & Rangari, V. D. (2014). A review on pharmacology and toxicology of *Elephantopus scaber* Linn. *Natural Product Research, 28*(11), 819-830.

Hisham, A., Pieters, I., Claeys, M., Dommisse, R., Berghe, D. V., & Vlietinck, A. (1992). Guaianolide Glucosides from *Elephantopus scaber*. *Planta Medica, 58*(5), 474-475.

Ho, W. Y., Ky, H., Yeap, S. K., Abdul Rahim, A., Omar, A. R., Ho, C. L., & Alitheen, N. B. (2009). Traditional practice, bioactivities and commercialization potential of *Elephantopus scaber* Linn. *Journal of Medicinal Plants Research, 3*(13), 1212-1221.

Ho, W. Y., Yeap, S. K., Ho, C. L., Abdul Rahim, R., & Alitheen, N. B. (2012). Hepatoprotective Activity of *Elephantopus scaber* on Alcohol-Induced Liver Damage in Mice. *Evidence Based Complementary Alternative Medicine, 2012*, 417953.

Hoffmann, C. (2000). COX-2 in brain and spinal cord implications for therapeutic use. *Current Medicinal Chemistry, 7*(11), 1113-1120.

Hoge, J., Yan, I., Janner, N., Schumacher, V., Chalaris, A., Steinmetz, O. M., . . . Mittrucker, H. W. (2013). IL-6 controls the innate immune response against Listeria monocytogenes via classical IL-6 signaling. *Journal of Immunology, 190*(2), 703-711.

Holcik, M., Gibson, H., & Korneluk, R. G. (2001). XIAP: apoptotic brake and promising therapeutic target. *Apoptosis, 6*(4), 253-261.

Hollenberg, S. M., & Cinel, I. (2009). Bench-to-bedside review: nitric oxide in critical illness--update 2008. *Critical Care, 13*(4), 218.

Hotchkiss, R. S., Strasser, A., McDunn, J. E., & Swanson, P. E. (2009). Cell death. *New England Journal of Medicine, 361*(16), 1570-1583.

Hsieh, H. L., & Yang, C. M. (2013). Role of redox signaling in neuroinflammation and neurodegenerative diseases. *BioMed Research International, 2013*, 484613.

Hsieh, M. J., Chien, S. Y., Chou, Y. E., Chen, C. J., Chen, J., & Chen, M. K. (2014). Hispolon from Phellinus linteus possesses mediate caspases activation and induces human nasopharyngeal carcinomas cells apoptosis through ERK1/2, JNK1/2 and p38 MAPK pathway. *Phytomedicine, 21*(12), 1746-1752.

Hsu, J. D., Kao, S. H., Ou, T. T., Chen, Y. J., Li, Y. J., & Wang, C. J. (2011). Gallic acid induces G2/M phase arrest of breast cancer cell MCF-7 through stabilization of p27(Kip1) attributed to disruption of p27(Kip1)/Skp2 complex. *Journal of Agriculture & Food Chemistry, 59*(5), 1996-2003.

Hu, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ullrich, A., & Schlessinger, J. (1992). Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Molecular and Cellular Biology, 12*(3), 981-990.

Huang, C. C., Lo, C. P., Chiu, C. Y., & Shyur, L. F. (2010). Deoxyelephantopin, a novel multifunctional agent, suppresses mammary tumour growth and lung metastasis and doubles survival time in mice. *British Journal of Pharmacology, 159*(4), 856-871.

Huang, T., Wu, X., Wang, Y., Ye, W. C., & Li, Y. L. (2009). Chemical Constituents of *Elephantopus scaber*. *Journal of Jinan University Natural Science & Medicine Edition, 30*(5), 553-555.

Hung, H. F., Hou, C. W., Chen, Y. L., Lin, C. C., Fu, H. W., Wang, J. S., & Jeng, K. C. (2011). *Elephantopus scaber* inhibits lipopolysaccharide-induced liver injury by suppression of signaling pathways in rats. *American Journal of Chinese Medicine, 39*(4), 705-717.

Hybertson, B. M., Gao, B., Bose, S. K., & McCord, J. M. (2011). Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. *Molecular Aspects of Medicine, 32*(4), 234-246.

Ichikawa, H., Nair, M. S., Takada, Y., Sheeja, D. B., Kumar, M. A., Oommen, O. V., & Aggarwal, B. B. (2006). Isodeoxyelephantopin, a novel sesquiterpene lactone, potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis through suppression of nuclear factor-kappaB (nf-kappaB) activation and nf-kappaB-regulated gene expression. *Clinical Cancer Research, 12*(19), 5910-5918.

Imai, K., & Yamamoto, H. (2008). Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis, 29*(4), 673-680.

Innamorato, N. G., Lastres-Becker, I., & Cuadrado, A. (2009). Role of microglial redox balance in modulation of neuroinflammation. *Current Opinion in Neurology, 22*(3), 308-314.

Janssen-Heininger, Y. M., Mossman, B. T., Heintz, N. H., Forman, H. J., Kalyanaraman, B., Finkel, T., . . . van der Vliet, A. (2008). Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radical Biology & Medicine, 45*(1), 1-17.

Jass, J. R. (2007). Molecular heterogeneity of colorectal cancer: Implications for cancer control. *Surgical Oncology, 16 Suppl 1*, S7-9.

Jayasooriya, R. G., Lee, K. T., Choi, Y. H., Moon, S. K., Kim, W. J., & Kim, G. Y. (2015). Antagonistic effects of acetylshikonin on LPS-induced NO and PGE2 production in BV2 microglial cells via inhibition of ROS/PI3K/Akt-mediated NF-kappaB signaling and activation of Nrf2-dependent HO-1. *In Vitro Cellular & Development Biology Animal, 51*(9), 975-986.

Jeffrey, K. L., Camps, M., Rommel, C., & Mackay, C. R. (2007). Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses. *Nature Reviews Drug Discovery, 6*(5), 391-403.

Jeong, J. W., Choi, I. W., Jo, G. H., Kim, G. Y., Kim, J., Suh, H., . . . Choi, Y. H. (2015). Anti-Inflammatory Effects of 3-(4'-Hydroxyl-3',5'-Dimethoxyphenyl)Propionic Acid, an Active Component of Korean Cabbage Kimchi, in Lipopolysaccharide-Stimulated BV2 Microglia. *Journal of Medicinal Food, 18*(6), 677-684.

Jin, Y. P., Fishbein, M. C., Said, J. W., Jindra, P. T., Rajalingam, R., Rozengurt, E., & Reed, E. F. (2004). Anti-HLA class I antibody-mediated activation of the PI3K/Akt signaling pathway and induction of Bcl-2 and Bcl-xL expression in endothelial cells. *Human Immunology, 65*(4), 291-302.

Joe, A. K., Liu, H., Suzui, M., Vural, M. E., Xiao, D., & Weinstein, I. B. (2002). Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clinical Cancer Research, 8*(3), 893-903.

Jost, P. J., & Ruland, J. (2007). Aberrant NF-kappaB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood, 109*(7), 2700-2707.

Jothy, S. L., Zakaria, Z., Chen, Y., Lau, Y. L., Latha, L. Y., & Sasidharan, S. (2011). Acute oral toxicity of methanolic seed extract of Cassia fistula in mice. *Molecules, 16*(6), 5268-5282.

Juan, M. E., Wenzel, U., Daniel, H., & Planas, J. M. (2008). Resveratrol induces apoptosis through ROS-dependent mitochondria pathway in HT-29 human colorectal carcinoma cells. *Journal of Agriculture and Food Chemistry, 56*(12), 4813-4818.

Jung, C. H., Jun, C. B., Ro, S. H., Kim, Y. M., Otto, N. M., Cao, J., . . . Kim, D. H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Molecular biology Cell, 20*(7), 1992-2003.

Jung, H. W., Chung, Y. S., Kim, Y. S., & Park, Y. K. (2007). Celastrol inhibits production of nitric oxide and proinflammatory cytokines through MAPK signal transduction and NF-kappaB in LPS-stimulated BV-2 microglial cells. *Experimental & Molecular Medicine, 39*(6), 715-721.

Kabeer, F. A., Sreedevi, G. B., Nair, M. S., Rajalekshmi, D. S., Gopalakrishnan, L. P., Kunjuraman, S., & Prathapan, R. (2013). Antineoplastic effects of deoxyelephantopin, a sesquiterpene lactone from *Elephantopus scaber*, on lung adenocarcinoma (A549) cells. *Journal Integrative Medicine, 11*(4), 269-277.

Kabeer, F. A., Sreedevi, G. B., Nair, M. S., Rajalekshmi, D. S., Gopalakrishnan, L. P., & Prathapan, R. (2014). Isodeoxyelephantopin from *Elephantopus scaber* (Didancao) induces cell cycle arrest and caspase-3-mediated apoptosis in breast carcinoma T47D cells and lung carcinoma A549 cells. *Chinese Medicine, 9*, 14.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., . . . Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO Journal, 19*(21), 5720-5728.

Kaltschmidt, B., Widera, D., & Kaltschmidt, C. (2005). Signaling via NF-kappaB in the nervous system. *Biochimica et Biophysica Acta, 1745*(3), 287-299.

Kaminska, B. (2005). MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. *Biochimica et Biophysica Acta, 1754*(1-2), 253-262.

Kang, C.-H., Choi, Y. H., Moon, S.-K., Kim, W.-J., & Kim, G.-Y. (2013). Quercetin inhibits lipopolysaccharide-induced nitric oxide production in BV2 microglial cells by suppressing the NF-κB pathway and activating the Nrf2-dependent HO-1 pathway. *International Immunopharmacology, 17*(3), 808-813.

Kanninen, K., Malm, T. M., Jyrkkanen, H. K., Goldsteins, G., Keksa-Goldsteine, V., Tanila, H., . . . Koistinaho, J. (2008). Nuclear factor erythroid 2-related factor 2 protects against beta amyloid. *Molecular Cell Neurosciences, 39*(3), 302-313.

Karin, M., Cao, Y., Greten, F. R., & Li, Z. W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nature Reviews Cancer, 2*(4), 301-310.

Karin, M., Yamamoto, Y., & Wang, Q. M. (2004). The IKK NF-kappa B system: a treasure trove for drug development. *Nature reviews Drug discovery, 3*(1), 17-26.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., & Waterfield, M. D. (2001). Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annual Review of Cell and Development Biology, 17*, 615-675.

Kelso, A. (1998). Cytokines: principles and prospects. *Immunology and Cell Biology, 76*(4), 300-317.

Khan, K. H., Yap, T. A., Yan, L., & Cunningham, D. (2013). Targeting the PI3K-AKT-mTOR signaling network in cancer. *Chinese Journal of Cancer, 32*(5), 253-265.

Khan, N., & Mukhtar, H. (2008). Multitargeted therapy of cancer by green tea polyphenols. *Cancer Letters, 269*(2), 269-280.

Kiefer, R., Lindholm, D., & Kreutzberg, G. W. (1993). Interleukin-6 and transforming growth factor-beta 1 mRNAs are induced in rat facial nucleus following motoneuron axotomy. *European Journal of Neuroscience, 5*(7), 775-781.

Kim, E. K., & Choi, E. J. (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochimica et Biophysica Acta, 1802*(4), 396-405.

Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology, 13*(2), 132-141.

Kim, S., Lee, T. J., Park, J. W., & Kwon, T. K. (2008). Overexpression of cFLIPs inhibits oxaliplatin-mediated apoptosis through enhanced XIAP stability and Akt activation in human renal cancer cells. *Journal of Cellular Biochemistry, 105*(4), 971-979.

Kim, Y. J., Hwang, S. Y., Oh, E. S., Oh, S., & Han, I. O. (2006). IL-1beta, an immediate early protein secreted by activated microglia, induces iNOS/NO in C6 astrocytoma cells through p38 MAPK and NF-kappaB pathways. *Journal of Neuroscience Research, 84*(5), 1037-1046.

Kim, Y. K., Na, K. S., Myint, A. M., & Leonard, B. E. (2016). The role of pro-inflammatory cytokines in neuroinflammation, neurogenesis and the neuroendocrine system in major depression. *Progress in Neuropsychopharmacology & Biological Psychiatry, 64*, 277-284.

Kimmelman, A. C. (2011). The dynamic nature of autophagy in cancer. *Genes & Development, 25*(19), 1999-2010.

Kischkel, F. C., Lawrence, D. A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., . . . Ashkenazi, A. (2001). Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *Journal of Biological Chemistry, 276*(49), 46639-46646.

Knowles, R. G., & Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochemical Journal, 298 (Pt 2)*, 249-258.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., . . . Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Molecular and Cellular Biology, 24*(16), 7130-7139.

Kolch, W. (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochemical Journal, 351 Pt 2*, 289-305.

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiological Reviews, 87*(1), 99-163.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., . . . Melino, G. (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death and Differentiation, 16*(1), 3-11.

Kroemer, G., & Jaattela, M. (2005). Lysosomes and autophagy in cell death control. *Nature Reviews Cancer, 5*(11), 886-897.

Krueger, A., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001). FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Molecular and Cellular Biology, 21*(24), 8247-8254.

Kulip, J. (2003). An ethnobotanical survey of medicinal and other useful plants of Muruts in Sabah, Malaysia. *Telopea, 10*(1), 81-98.

Kumar, B., Vijayakumar, M., Govindarajan, R., & Pushpangadan, P. (2007). Ethnopharmacological approaches to wound healing--exploring medicinal plants of India. *Journal of Ethnopharmacology, 114*(2), 103-113.

Kumar, D., Shankar, S., & Srivastava, R. K. (2014). Rottlerin induces autophagy and apoptosis in prostate cancer stem cells via PI3K/Akt/mTOR signaling pathway. *Cancer Letters, 343*(2), 179-189.

Kumar, S. S., Perumal, P., & Suresh, B. (2004). Antibacterial studies on leaf extract of *Elephantopus scaber* Linn. *Ancient Science Life, 23*(3), 6-8.

Kuo, Y. F., Su, Y. Z., Tseng, Y. H., Wang, S. Y., Wang, H. M., & Chueh, P. J. (2010). Flavokawain B, a novel chalcone from Alpinia pricei Hayata with potent apoptotic activity: Involvement of ROS and GADD153 upstream of mitochondria-dependent apoptosis in HCT116 cells. *Free Radic Biol Med, 49*(2), 214-226.

Kurutas, E. B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition Journal, 15*(1), 71.

Kushima, Y., & Hatanaka, H. (1992). Interleukin-6 and leukemia inhibitory factor promote the survival of acetylcholinesterase-positive neurons in culture from embryonic rat spinal cord. *Neuroscience Letters, 143*(1-2), 110-114.

Kuwano, A., Ikeda, H., Takeda, K., Nakai, H., Kondo, I., & Shibahara, S. (1994). Mapping of the human gene for inducible heme oxygenase to chromosome 22q12. *Tohoku Journal of Experimental Medicine, 172*(4), 389-392.

Labi, V., & Erlacher, M. (2015). How cell death shapes cancer. *Cell Death & Disease, 6*, e1675.

Lamkanfi, M., & Dixit, V. M. (2012). Inflammasomes and their roles in health and disease. *Annual Review of Cell and Development Biology, 28*, 137-161.

Landi, F., Cesari, M., Onder, G., Russo, A., Torre, S., & Bernabei, R. (2003). Non-steroidal anti-inflammatory drug (NSAID) use and Alzheimer disease in community-dwelling elderly patients. *American Journal of Geriatric Psychiatry, 11*(2), 179-185.

Lavrik, I. N., Golks, A., & Krammer, P. H. (2005). Caspases: pharmacological manipulation of cell death. *Journal of Clinical Investigation, 115*(10), 2665-2672.

Lawes, D., & Boulos, P. B. (2002). Advances in the management of rectal cancer. *Journal of Royal Society of Medicine, 95*(12), 587-590.

Lawrence, M. C., Jivan, A., Shao, C., Duan, L., Goad, D., Zaganjor, E., . . . Cobb, M. H. (2008). The roles of MAPKs in disease. *Cell Research, 18*(4), 436-442.

Ledoux, A. C., & Perkins, N. D. (2014). NF-kappaB and the cell cycle. *Biochemical Society Transactions, 42*(1), 76-81.

Lee, C., Park, G. H., Lee, S. R., & Jang, J. H. (2013). Attenuation of beta-amyloid-induced oxidative cell death by sulforaphane via activation of NF-E2-related factor 2. *Oxidative Medicine Cellular Longevity, 2013*, 313510.

Lee, J. W., Bae, C. J., Choi, Y. J., Kim, S. I., Kwon, Y. S., Lee, H. J., . . . Chun, W. (2014). 3,4,5-trihydroxycinnamic acid inhibits lipopolysaccharide (LPS)-induced inflammation by Nrf2 activation *in vitro* and improves survival of mice in LPS-induced endotoxemia model *in vivo*. *Molecular and Cellular Biochemistry, 390*(1-2), 143-153.

Lee, S., Cho, N. Y., Choi, M., Yoo, E. J., Kim, J. H., & Kang, G. H. (2008). Clinicopathological features of CpG island methylator phenotype-positive colorectal cancer and its adverse prognosis in relation to KRAS/BRAF mutation. *Pathology International, 58*(2), 104-113.

Lee, S. H., & Hannink, M. (2002). Characterization of the nuclear import and export functions of Ikappa B(epsilon). *Journal of Biological Chemistry, 277*(26), 23358-23366.

Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., . . . Yoo, N. J. (2001). Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma. *Oncogene, 20*(3), 399-403.

Lee, W. L., & Shyur, L. F. (2012). Deoxyelephantopin impedes mammary adenocarcinoma cell motility by inhibiting calpain-mediated adhesion dynamics

and inducing reactive oxygen species and aggresome formation. *Free Radical Biology & Medicine, 52*(8), 1423-1436.

Lee, Y. S., Choi, K. M., Kim, W., Jeon, Y. S., Lee, Y. M., Hong, J. T., Yun, Y. P., & Yoo, H. S. (2013). Hinokitiol inhibits cell growth through induction of S-phase arrest and apoptosis in human colon cancer cells and suppresses tumor growth in a mouse xenograft experiment. *Journal of Natural Products, 76*(12), 2195-2202.

Leonti, M., & Casu, L. (2013). Traditional medicines and globalization: current and future perspectives in ethnopharmacology. *Frontiers in Pharmacology, 4*, 92.

Levine, B., & Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell, 132*(1), 27-42.

Li-Weber, M. (2013). Targeting apoptosis pathways in cancer by Chinese medicine. *Cancer Letters, 332*(2), 304-312.

Li, D. M., & Sun, H. (1997). TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Research, 57*(11), 2124-2129.

Li, H. Y., Zhang, J., Sun, L. L., Li, B. H., Gao, H. L., Xie, T., . . . Ye, Z. M. (2015). Celastrol induces apoptosis and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells: an *in vitro* and *in vivo* study. *Cell Death & Disease, 6*, e1604.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., . . . Parsons, R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science, 275*(5308), 1943-1947.

Li, L. K., Rola, A. S., Kaid, F. A., Ali, A. M., & Alabsi, A. M. (2016). Goniothalamin induces cell cycle arrest and apoptosis in H400 human oral squamous cell carcinoma: A caspase-dependent mitochondrial-mediated pathway with downregulation of NF-kappabeta. *Archives of Oral Biology, 64*, 28-38.

Li, X., & Darzynkiewicz, Z. (2000). Cleavage of Poly(ADP-ribose) polymerase measured in situ in individual cells: relationship to DNA fragmentation and cell cycle position during apoptosis. *Experimental Cell Research, 255*(1), 125-132.

Li, X., Xu, H. L., Liu, Y. X., An, N., Zhao, S., & Bao, J. K. (2013). Autophagy modulation as a target for anticancer drug discovery. *Acta Pharmacologica Sinica, 34*(5), 612-624.

Liang, Q. L., Min, Z. D., & Tang, Y. P. (2008). A new elemanolide sesquiterpene lactone from *Elephantopus scaber*. *Journal of Asian Natural Products Research, 10*(5-6), 403-407.

Liang, Q. L., Gong, Z. N., & Min, Z.D. (2007). Studies on Triterpenes from *Elephantopus scaber*. *Chinese pharmaceutical journal*(7), 494-496.

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development, 140*(15), 3079-3093.

Limón-Pacheco, J., & Gonsebatt, M. E. (2009). The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 674*(1), 137-147.

Lin, A. (2003). Activation of the JNK signaling pathway: breaking the brake on apoptosis. *Bioessays, 25*(1), 17-24.

Lin, C.-C., Tsai, C.-C., & Yen, M.-H. (1995). The evaluation of hepatoprotective effects of Taiwan folk medicine 'Teng-Khia-U'. *Journal of Ethnopharmacology, 45*(2), 113-123.

Liu, B., Gao, H. M., Wang, J. Y., Jeohn, G. H., Cooper, C. L., & Hong, J. S. (2002). Role of nitric oxide in inflammation ‐ mediated neurodegeneration. *Annals of New York Academy of Science, 962*(1), 318-331.

Liu, B., & Hong, J. S. (2003). Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *Journal of Pharmacology Experimental Therapeutics, 304*(1), 1-7.

Liu, J., Garcia-Cardena, G., & Sessa, W. C. (1995). Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry, 34*(38), 12333-12340.

Lo, J. Y., Kamarudin, M. N., Hamdi, O. A., Awang, K., & Kadir, H. A. (2015). Curcumenol isolated from Curcuma zedoaria suppresses Akt-mediated NF-kappaB activation and p38 MAPK signaling pathway in LPS-stimulated BV-2 microglial cells. *Food & Function, 6*(11), 3550-3559.

Lopez, J., & Tait, S. W. (2015). Mitochondrial apoptosis: killing cancer using the enemy within. *British Journal of Cancer, 112*(6), 957-962.

Lowe, S. W., & Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis, 21*(3), 485-495.

Lubos, E., Loscalzo, J., & Handy, D. E. (2011). Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxidant & Redox Signaling, 15*(7), 1957-1997.

Lue, L. F., Rydel, R., Brigham, E. F., Yang, L. B., Hampel, H., Murphy, G. M., Jr., . . . Rogers, J. (2001). Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia *in vitro*. *Glia, 35*(1), 72-79.

Lundberg, J. O., Gladwin, M. T., & Weitzberg, E. (2015). Strategies to increase nitric oxide signalling in cardiovascular disease. *Nature reviews Drug discovery, 14*(9), 623-641.

Lushchak, V. I. (2014). Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-Biological Interactions, 224*, 164-175.

Lyss, G., Knorre, A., Schmidt, T. J., Pahl, H. L., & Merfort, I. (1998). The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. *Journal of Biological Chemistry, 273*(50), 33508-33516.

Ma, C. X., Reinert, T., Chmielewska, I., & Ellis, M. J. (2015). Mechanisms of aromatase inhibitor resistance. *Nature Reviews Cancer, 15*(5), 261-275.

Ma, X. M., & Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nature Reviews Molecular Cell Biology, 10*(5), 307-318.

Maiuri, M. C., Zalckvar, E., Kimchi, A., & Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature Reviews Molecular Cell Biology, 8*(9), 741-752.

Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews Cancer, 9*(3), 153-166.

Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell, 129*(7), 1261-1274.

Marchant, A., Bruyns, C., Vandenabeele, P., Ducarme, M., Gerard, C., Delvaux, A., . . . Goldman, M. (1994). Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia. *European Journal of Immunology, 24*(5), 1167-1171.

Markesbery, W. R. (1997). Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Biology & Medicine, 23*(1), 134-147.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., . . . Vogelstein, B. (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science, 268*(5215), 1336-1338.

Martel, C., Huynh le, H., Garnier, A., Ventura-Clapier, R., & Brenner, C. (2012). Inhibition of the Mitochondrial Permeability Transition for Cytoprotection: Direct versus Indirect Mechanisms. *Biochemistry Research International, 2012*, 213403.

Martin, R. M., Leonhardt, H., &Cardoso, M. C. (2005). DNA labeling in living cells. *Cytometry A, 67*(1), 45-52.

Martinou, J. C., & Youle, R. J. (2011). Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Developmental Cell, 21*(1), 92-101.

Matsumoto, G., Wada, K., Okuno, M., Kurosawa, M., & Nukina, N. (2011). Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Molecular Cell, 44*(2), 279-289.

Mattson, M. P., & Camandola, S. (2001). NF-kappaB in neuronal plasticity and neurodegenerative disorders. *Journal of Clinical Investigation, 107*(3), 247-254.

McCoy, M. K., & Tansey, M. G. (2008). TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *J Neuroinflammation, 5*, 45.

McCubrey, J. A., Lahair, M. M., & Franklin, R. A. (2006). Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxidant & Redox Signaling, 8*(9-10), 1775-1789.

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology, 5*(4), a008656.

Mehmood, T., Maryam, A., Zhang, H., Li, Y., Khan, M., & Ma, T. (2017). Deoxyelephantopin induces apoptosis in HepG2 cells via oxidative stress, NF-kappaB inhibition and mitochondrial dysfunction. *Biofactors, 43*(1), 63-72.

Mehrpour, M., Esclatine, A., Beau, I., & Codogno, P. (2010). Autophagy in health and disease. 1. Regulation and significance of autophagy: an overview. *American Journal of Physiology Cell Physiology, 298*(4), C776-785.

Meijer, A. J., & Codogno, P. (2009). Autophagy: regulation and role in disease. *Critical Reviews in Clinical Labaratory Sciences, 46*(4), 210-240.

Mellor, P., Furber, L. A., Nyarko, J. N., & Anderson, D. H. (2012). Multiple roles for the p85alpha isoform in the regulation and function of PI3K signalling and receptor trafficking. *Biochemical Journal, 441*(1), 23-37.

Mi, Y., Xiao, C., Du, Q., Wu, W., Qi, G., & Liu, X. (2016). Momordin Ic couples apoptosis with autophagy in human hepatoblastoma cancer cells by reactive oxygen species (ROS)-mediated PI3K/Akt and MAPK signaling pathways. *Free Radical Biology & Medicine, 90*, 230-242.

Milhas, D., Cuvillier, O., Therville, N., Clave, P., Thomsen, M., Levade, T., . . . Segui, B. (2005). Caspase-10 triggers Bid cleavage and caspase cascade activation in FasL-induced apoptosis. *Journal of Biological Chemistry, 280*(20), 19836-19842.

Miller, A. H., Maletic, V., & Raison, C. L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biological Psychiatry, 65*(9), 732-741.

Miller, C., & Koeffler, H. P. (1993). P53 mutations in human cancer. *Leukemia, 7 Suppl 2*, S18-21.

Miller, K. D., Siegel, R. L., Lin, C. C., Mariotto, A. B., Kramer, J. L., Rowland, J. H., . . . Jemal, A. (2016). Cancer treatment and survivorship statistics, 2016. *CA Cancer Journal Clinicians, 66*(4), 271-289.

Minghetti, L. (2004). Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *Journal of Neuropathology and Experimental Neurology, 63*(9), 901-910.

Mishra, J., Drummond, J., Quazi, S. H., Karanki, S. S., Shaw, J. J., Chen, B., & Kumar, N. (2013). Prospective of colon cancer treatments and scope for combinatorial

approach to enhanced cancer cell apoptosis. *Critical Reviews in Oncology Hematology, 86*(3), 232-250.

Mizushima, N. (2007). Autophagy: process and function. *Genes and Development, 21*(22), 2861-2873.

Moi, P., Chan, K., Asunis, I., Cao, A., & Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of National Academy of Sciences United States of America, 91*(21), 9926-9930.

Molinari, F., & Frattini, M. (2013). Functions and Regulation of the PTEN Gene in Colorectal Cancer. *Frontiers in Oncology, 3*, 326.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science Technology, 26*(2), 211-219.

Montgomery, S. L., Narrow, W. C., Mastrangelo, M. A., Olschowka, J. A., O'Banion, M. K., & Bowers, W. J. (2013). Chronic neuron- and age-selective down-regulation of TNF receptor expression in triple-transgenic Alzheimer disease mice leads to significant modulation of amyloid- and Tau-related pathologies. *American Journal of Pathology, 182*(6), 2285-2297.

Moorehead, R. A., Hojilla, C. V., De Belle, I., Wood, G. A., Fata, J. E., Adamson, E. D., . . . Khokha, R. (2003). Insulin-like growth factor-II regulates PTEN expression in the mammary gland. *Journal of Biological Chemistry, 278*(50), 50422-50427.

Morita, I. (2002). Distinct functions of COX-1 and COX-2. *Prostaglandins Other Lipid Mediat, 68-69*, 165-175.

Muller, P. A., & Vousden, K. H. (2013). p53 mutations in cancer. *Nature Cell Biology, 15*(1), 2-8.

Munoz, L., & Ammit, A. J. (2010). Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. *Neuropharmacology, 58*(3), 561-568.

Nair, S., Doh, S. T., Chan, J. Y., Kong, A. N., & Cai, L. (2008). Regulatory potential for concerted modulation of Nrf2- and Nfkb1-mediated gene expression in inflammation and carcinogenesis. *British Journal of Cancer, 99*(12), 2070-2082.

Nakamura, T., & Lipton, S. A. (2011). S-nitrosylation of critical protein thiols mediates protein misfolding and mitochondrial dysfunction in neurodegenerative diseases. *Antioxidant & Redox Signaling, 14*(8), 1479-1492.

Nathan, C. (2002). Points of control in inflammation. *Nature, 420*(6917), 846-852.

Netea, M. G., Simon, A., van de Veerdonk, F., Kullberg, B. J., Van der Meer, J. W., & Joosten, L. A. (2010). IL-1beta processing in host defense: beyond the inflammasomes. *PLoS Pathogens, 6*(2), e1000661.

Netter, J., Douard, R., Durdux, C., Landi, B., Berger, A., & Taieb, J. (2016). Advances in management of adjuvant chemotherapy in rectal cancer: Consequences for clinical practice. *Clinics and Research in Hepatology and Gastroenterology, 40*(5), 546-552.

Nguyen, T., Nioi, P., & Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *Journal of Biological Chemistry, 284*(20), 13291-13295.

Nguyen, T. H., Mustafa, F. B., Pervaiz, S., Ng, F. S., & Lim, L. H. (2008). ERK1/2 activation is required for resveratrol-induced apoptosis in MDA-MB-231 cells. *International Journal of Oncology, 33*(1), 81-92.

Ni, N. T., Fotso, S., Sevvana, M., Sheldrick, G. M., Fiebig, H. H., Kelter, G., & Laatsch, H. (2005). Sesquiterpene lactones from *Elephantopus scaber*. Zeitschrift für Naturforschung, *60b*, 200-204.

Nikoletopoulou, V., Markaki, M., Palikaras, K., & Tavernarakis, N. (2013). Crosstalk between apoptosis, necrosis and autophagy. *Biochimica et Biophysica Acta, 1833*(12), 3448-3459.

Nimse, S. B., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *Royal Society of Chemistry Advances, 5*, 27986-28006.

Nyberg, K. A., Michelson, R. J., Putnam, C. W., & Weinert, T. A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. *Annual Review of Genetics, 36*, 617-656.

Ogryzko, V. V., Wong, P., & Howard, B. H. (1997). WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Molecular and Cellular Biology, 17*(8), 4877-4882.

Okkenhaug, K., & Vanhaesebroeck, B. (2003). PI3K in lymphocyte development, differentiation and activation. *Nature Review Immunology, 3*(4), 317-330.

Okouchi, M., Ekshyyan, O., Maracine, M., & Aw, T. Y. (2007). Neuronal apoptosis in neurodegeneration. *Antioxidant & Redox Signaling, 9*(8), 1059-1096.

Olivier, M., Hollstein, M., & Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspective Biology, 2*(1), a001008.

Olsson, M., & Zhivotovsky, B. (2011). Caspases and cancer. *Cell Death and Differentiation, 18*(9), 1441-1449.

Oren, M., & Bartek, J. (2007). The sunny side of p53. *Cell, 128*(5), 826-828.

Ortega, S., Malumbres, M., & Barbacid, M. (2002). Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochimica et Biophysica Acta, 1602*(1), 73-87.

Osaki, M., Oshimura, M., & Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis, 9*(6), 667-676.

Otterbein, L. E., Soares, M. P., Yamashita, K., & Bach, F. H. (2003). Heme oxygenase-1: unleashing the protective properties of heme. *Trends in Immunology, 24*(8), 449-455.

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. *Nature Reviews Cancer, 17*(2), 93-115.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation, 45*(6), 487-498.

Paine, A., Eiz-Vesper, B., Blasczyk, R., & Immenschuh, S. (2010). Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochemical Pharmacology, 80*(12), 1895-1903.

Papadatos-Pastos, D., Rabbie, R., Ross, P., & Sarker, D. (2015). The role of the PI3K pathway in colorectal cancer. *Critical Reviews in Oncology Hematology, 94*(1), 18-30.

Park, J., Min, J. S., Kim, B., Chae, U. B., Yun, J. W., Choi, M. S., . . . Lee, D. S. (2015). Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-kappaB pathways. *Neuroscience Letters, 584*, 191-196.

Pelicano, H., Carney, D., & Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates, 7*(2), 97-110.

Pereira, L., Mariadason, J. M., Hannan, R. D., & Dhillon, A. S. (2015). Implications of epithelial-mesenchymal plasticity for heterogeneity in colorectal cancer. *Frontiers in Oncology, 5*, 13.

Perkins, N. D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nature Reviews Molecular Cell Biology, 8*(1), 49-62.

Phipps, R. P., Stein, S. H., & Roper, R. L. (1991). A new view of prostaglandin E regulation of the immune response. *Immunology Today, 12*(10), 349-352.

Pisoschi, A. M., & Pop, A. (2015). The role of antioxidants in the chemistry of oxidative stress: A review. *European Journal of Medicinal Chemistry, 97*, 55-74.

Pitchai, D., Roy, A., & Ignatius, C. (2014). *In vitro* evaluation of anticancer potentials of lupeol isolated from *Elephantopus scaber* L. on MCF-7 cell line. *Journal of Advanced Pharmaceutical Technology & Research, 5*(4), 179-184.

Platanias, L. C. (2003). Map kinase signaling pathways and hematologic malignancies. *Blood, 101*(12), 4667-4679.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., & Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & Development, 8*(1), 9-22.

Porta, C., Paglino, C., & Mosca, A. (2014). Targeting PI3K/Akt/mTOR Signaling in Cancer. *Frontiers in Oncology, 4*, 64.

Pradelli, L. A., Beneteau, M., & Ricci, J. E. (2010). Mitochondrial control of caspase-dependent and -independent cell death. *Cellular and Molecular Life Sciences, 67*(10), 1589-1597.

Prasad, S., Madan, E., Nigam, N., Roy, P., George, J., & Shukla, Y. (2009). Induction of apoptosis by lupeol in human epidermoid carcinoma A431 cells through regulation of mitochondrial, Akt/PKB and NFkappaB signaling pathways. *Cancer Biology & Therapy, 8*(17), 1632-1639.

Prehn, J. H., & Krieglstein, J. (1993). Platelet-activating factor antagonists reduce excitotoxic damage in cultured neurons from embryonic chick telencephalon and protect the rat hippocampus and neocortex from ischemic injury *in vivo*. *Journal of Neuroscience Research, 34*(2), 179-188.

Querfurth, H. W., & LaFerla, F. M. (2010). Alzheimer's disease. *New England Journal of Medicine, 362*(4), 329-344.

Rabe, B., Chalaris, A., May, U., Waetzig, G. H., Seegert, D., Williams, A. S., . . . Scheller, J. (2008). Transgenic blockade of interleukin 6 transsignaling abrogates inflammation. *Blood, 111*(3), 1021-1028.

Rajesh, M., &Latha, M. (2001). Hepatoprotection by *Elephantopus scaber* Linn. in CCl4-induced liver injury. *Indian Journal of Physiology and Pharmacology, 45*(4), 481-486.

Ramsey, C. P., Glass, C. A., Montgomery, M. B., Lindl, K. A., Ritson, G. P., Chia, L. A., . . . Jordan-Sciutto, K. L. (2007). Expression of Nrf2 in neurodegenerative diseases. *Journal of Neuropathology & Experimental Neurology, 66*(1), 75-85.

Ranjan, A., & Srivastava, S. K. (2016). Penfluridol suppresses pancreatic tumor growth by autophagy-mediated apoptosis. *Scientific Reports, 6*, 26165.

Rasoanaivo, P., Petitjean, A., Ratsimamanga, U. S., & Rakoto, R. A. (1992). Medicinal plants used to treat malaria in Madagascar. *Journal of Ethnopharmacology, 37*(2), 117-127.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *Journal of Cell Biology, 124*(1-2), 1-6.

Rivlin, N., Brosh, R., Oren, M., & Rotter, V. (2011). Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes Cancer, 2*(4), 466-474.

Rogov, V., Dotsch, V., Johansen, T., & Kirkin, V. (2014). Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Molecular Cell, 53*(2), 167-178.

Roninson, I. B. (2003). Tumor cell senescence in cancer treatment. *Cancer Research, 63*(11), 2705-2715.

Rosales, C. S., Reiter, R. J., Tan, D. X., Ortiz, G. G., & Lopez, A. G. (2010). Functional aspects of redox control during neuroinflammation. *Antioxidant & Redox Signaling, 13*(2), 193-247.

Rosenberger, J., Petrovics, G., & Buzas, B. (2001). Oxidative stress induces proorphanin FQ and proenkephalin gene expression in astrocytes through p38- and ERK-MAP kinases and NF-kappaB. *Journal of Neurochemistry, 79*(1), 35-44.

Ross, C. A., & Poirier, M. A. (2005). Opinion: What is the role of protein aggregation in neurodegeneration? *Nature Reviews Molecular Cell Biology, 6*(11), 891-898.

Roux, P. P., & Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and Molecular Biology Reviews, 68*(2), 320-344.

Rubio, P. J. M., & Morillas, R. J. M. (2012). A review: inflammatory process in Alzheimer's disease, role of cytokines. *ScientificWorldJournal, 2012*, 756357.

Russo, M. V., & McGavern, D. B. (2016). Inflammatory neuroprotection following traumatic brain injury. *Science, 353*(6301), 783-785.

Sagar, R., & Sahoo, H. B. (2012). Evaluation of antiasthmatic activity of ethanolic extract of *Elephantopus scaber* L. leaves. *Indian Journal of Pharmacology, 44*(3), 398-401.

Saha, R. N., & Pahan, K. (2006). Regulation of inducible nitric oxide synthase gene in glial cells. *Antioxidant & Redox Signaling, 8*(5-6), 929-947.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., . . . Miller, L. L. (2000). Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *New England Journal of Medicine, 343*(13), 905-914.

Salvioli, S., Barbi, C., Dobrucki, J., Moretti, L., Pinti, M., Pedrazzi, J., . . . Cossarizza, A. (2000). Opposite role of changes in mitochondrial membrane potential in different apoptotic processes. *FEBS Letters, 469*(2-3), 186-190.

Sanchez, L. E., Flashner, A. E., Shalapour, S., Zhong, Z., Taniguchi, K., Levitzki, A., & Karin, M. (2016). Targeting colorectal cancer via its microenvironment by inhibiting IGF-1 receptor-insulin receptor substrate and STAT3 signaling. *Oncogene, 35*(20), 2634-2644.

Sanchez, P. I., Murguia, J. R., & Perona, R. (1998). Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene, 16*(4), 533-540.

Santhosh, K. S., Subramanian, A., Uma, C., Rengaiyan, R., Sambandan, K., Sekar, K. G., Sudarshan, M., & Chakraborty, A. (2012). Trace elemental analysis and antimicrobial activities of *Elephantopus scaber* L. *Journal of Phytology, 4*(4), 8-13.

Sarbassov, D. D., Guertin, D. A., Ali, S. M., & Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science, 307*(5712), 1098-1101.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., . . . Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO Journal, 17*(6), 1675-1687.

Scandalios, J. G. (1993). Oxygen stress and superoxide dismutases. *Plant Physiology, 101*(1), 7.

Schutte, B., Nieland, L., van Engeland, M., Henfling, M. E., Meijer, L., & Ramaekers, F. C. (1997). The effect of the cyclin-dependent kinase inhibitor olomoucine on cell cycle kinetics. *Experimental Cell Research, 236*(1), 4-15.

Seger, R., & Krebs, E. G. (1995). The MAPK signaling cascade. *Federation of American Societies for Experimental Biology Journal, 9*(9), 726-735.

Sen, R., & Baltimore, D. (1986). Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell, 47*(6), 921-928.

Sen, S., & Chakraborty, R. (2011). The Role of Antioxidants in Human Health. *American Chemical Society*, 1-37.

Ser, H. L., Palanisamy, U. D., Yin, W. F., Abd Malek, S. N., Chan, K. G., Goh, B. H., & Lee, L. H. (2015). Presence of antioxidative agent, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- in newly isolated Streptomyces mangrovisoli sp. nov. *Frontiers in Microbiology, 6*, 854.

Serhan, C. N., & Savill, J. (2005). Resolution of inflammation: the beginning programs the end. *Nature Immunology, 6*(12), 1191-1197.

Shabab, T., Khanabdali, R., Moghadamtousi, S. Z., Kadir, H. A., & Mohan, G. (2017). Neuroinflammation pathways: a general review. *International Journal of Neuroscience, 127*(7), 624-633.

Sherr, C. J., & Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Development, 13*(12), 1501-1512.

Shi, Q., & Gibson, G. E. (2007). Oxidative stress and transcriptional regulation in Alzheimer disease. *Alzheimer Disease and Associated Disorders, 21*(4), 276-291.

Shih, R. H., Wang, C. Y., & Yang, C. M. (2015). NF-kappaB Signaling Pathways in Neurological Inflammation: A Mini Review. *Frontiers in Molecular Neuroscience, 8*, 77.

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA Cancer Journal Clinicians, 66*(1), 7-30.

Sim, K. Y., & Lee, H.T. (1969). Constituents of *Elephantopus scaber* (Compositae). *Phytochemistry, 8*(5), 933-934.

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis, 5*(5), 415-418.

Simon, L. S. (1999). Role and regulation of cyclooxygenase-2 during inflammation. *American Journal of Medicine, 106*(5), 37S-42S.

Singh, S. D., Krishna, V., Mankani, K. L., Manjunatha, B. K., Vidya, S. M., & Manohara, Y. N. (2005). Wound healing activity of the leaf extracts and deoxyelephantopin isolated from *Elephantopus scaber* Linn. *Indian Journal of Pharmacology, 37*(4), 238-242.

Singleton, J. W., & Laster, L. (1965). Biliverdin reductase of guinea pig liver. *Journal of Biological Chemistry, 240*(12), 4780-4789.

Slee, E. A., Adrain, C., & Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *Journal of Biological Chemistry, 276*(10), 7320-7326.

Smith, J. A., Das, A., Ray, S. K., & Banik, N. L. (2012). Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Research Bulletin, 87*(1), 10-20.

Society, A. C. (2017). *Cancer Facts & Figures 2017*. Atlanta: American Cancer Society.

Sosa, V., Moline, T., Somoza, R., Paciucci, R., Kondoh, H., & Me, L. L. (2013). Oxidative stress and cancer: an overview. *Ageing Research Reviews, 12*(1), 376-390.

Souza, R. F., Appel, R., Yin, J., Wang, S., Smolinski, K. N., Abraham, J. M., . . . Meltzer, S. J. (1996). Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. *Nature Genetics, 14*(3), 255-257.

Srisook, K., & Cha, Y. N. (2005). Super-induction of HO-1 in macrophages stimulated with lipopolysaccharide by prior depletion of glutathione decreases iNOS expression and NO production. *Nitric Oxide, 12*(2), 70-79.

Stadtman, E. R. (2004). Role of oxidant species in aging. *Current Medicinal Chemistry, 11*(9), 1105-1112.

Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., . . . Mak, T. W. (2001). Regulation of PTEN transcription by p53. *Molecular Cell, 8*(2), 317-325.

Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., . . . DeFranco, D. B. (2000). Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *Journal of Biological Chemistry, 275*(16), 12200-12206.

Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., . . . Hawkins, P. T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science, 277*(5325), 567-570.

Strasser, A., Huang, D. C., & Vaux, D. L. (1997). The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumourigenesis and resistance to chemotherapy. *Biochimica et Biophysica Acta, 1333*(2), F151-178.

Stratikopoulos, E. E., & Parsons, R. E. (2016). Molecular Pathways: Targeting the PI3K Pathway in Cancer-BET Inhibitors to the Rescue. *Clinical Cancer Research, 22*(11), 2605-2610.

Streit, W. J., & Xue, Q. S. (2010). The Brain's Aging Immune System. *Aging and Disease, 1*(3), 254-261.

Su, C. C., Lin, J. G., Li, T. M., Chung, J. G., Yang, J. S., Ip, S. W., . . . Chen, G. W. (2006). Curcumin-induced apoptosis of human colon cancer colo 205 cells through the production of ROS, Ca2+ and the activation of caspase-3. *Anticancer Research, 26*(6B), 4379-4389.

Su, M., Chung, H. Y., & Li, Y. (2011). Deoxyelephantopin from *Elephantopus scaber* L. induces cell-cycle arrest and apoptosis in the human nasopharyngeal cancer CNE cells. *Biochemical and Biophysical Research Communications, 411*(2), 342-347.

Su, M., Wu, X., Chung, H. Y., Li, Y., & Ye, W. (2009). Antiproliferative activities of five Chinese medicinal herbs and active compounds in *Elephantopus scaber*. *Natural product communications, 4*(8), 1025-1030.

Subramaniam, S., Zirrgiebel, U., von Bohlen Und Halbach, O., Strelau, J., Laliberte, C., Kaplan, D. R., & Unsicker, K. (2004). ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. *Journal of Cell Biology, 165*(3), 357-369.

Sun, G. Y., Chen, Z., Jasmer, K. J., Chuang, D. Y., Gu, Z., Hannink, M., & Simonyi, A. (2015). Quercetin Attenuates Inflammatory Responses in BV-2 Microglial Cells: Role of MAPKs on the Nrf2 Pathway and Induction of Heme Oxygenase-1. *PLoS One, 10*(10), e0141509.

Surh, Y.J., & Na, H. K. (2008). NF-κB and Nrf2 as prime molecular targets for chemoprevention and cytoprotection with anti-inflammatory and antioxidant phytochemicals. *Genes & Nutrition, 2*(4), 313-317.

Suzuki, M., Youle, R. J., & Tjandra, N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell, 103*(4), 645-654.

Tait, S. W., & Green, D. R. (2013). Mitochondrial regulation of cell death. *Cold Spring Harbor Perspectives in Biology, 5*(9).

Tak, P. P., & Firestein, G. S. (2001). NF-κB: a key role in inflammatory diseases. *Journal of Clinical Investigation, 107*(1), 7-11.

Talevi, A. (2015). Multi-target pharmacology: possibilities and limitations of the "skeleton key approach" from a medicinal chemist perspective. *Frontiers in Pharmacology, 6*, 205.

Tartaglia, L. A., & Goeddel, D. V. (1992). Two TNF receptors. *Immunology Today, 13*(5), 151-153.

Teiten, M. H., Eifes, S., Dicato, M., & Diederich, M. (2010). Curcumin-the paradigm of a multi-target natural compound with applications in cancer prevention and treatment. *Toxins (Basel), 2*(1), 128-162.

Terashima, M., Kitada, K., Ochiai, A., Ichikawa, W., Kurahashi, I., Sakuramoto, S., . . . Sasako, M. (2012). Impact of expression of human epidermal growth factor receptors EGFR and ERBB2 on survival in stage II/III gastric cancer. *Clinical Cancer Research, 18*(21), 5992-6000.

Than, N. N., Fotso, S., Sevvana, M., Sheldrick, G. M., Fiebig, H. H., Kelter, G., & Laatsch, H. (2005). Sesquiterpene lactones from *Elephantopus scaber*. *Zeitschrift für Naturforschung, 60*, 200-204.

Thiabaud, G., Pizzocaro, S., Garcia, S. R., Latour, J. M., Monzani, E., & Casella, L. (2013). Heme binding induces dimerization and nitration of truncated beta-amyloid peptide Abeta16 under oxidative stress. *Angewandte Chemie International Edition England, 52*(31), 8041-8044.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., & Biswal, S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Research, 62*(18), 5196-5203.

Thorpe, L. M., Yuzugullu, H., & Zhao, J. J. (2015). PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nature Reviews Cancer, 15*(1), 7-24.

Tilg, H., Dinarello, C. A., & Mier, J. W. (1997). IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunology Today, 18*(9), 428-432.

Tong, J. S., Zhang, Q. H., Huang, X., Fu, X. Q., Qi, S. T., Wang, Y. P., . . . Sun, Q. Y. (2011). Icaritin causes sustained ERK1/2 activation and induces apoptosis in human endometrial cancer cells. *PLoS One, 6*(3), e16781.

Tong, K. I., Padmanabhan, B., Kobayashi, A., Shang, C., Hirotsu, Y., Yokoyama, S., & Yamamoto, M. (2007). Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Molecular and Cellular Biology, 27*(21), 7511-7521.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet, T. J., & Jemal, A. (2015). Global cancer statistics, 2012. *CA Cancer Journal Clinicians, 65*(2), 87-108.

Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature Reviews Drug Discovery, 8*(7), 579-591.

Tsujimoto, Y., & Shimizu, S. (2005). Another way to die: autophagic programmed cell death. *Cell Death and Differentiation, 12 Suppl 2*, 1528-1534.

Tuppo, E. E., & Arias, H. R. (2005). The role of inflammation in Alzheimer's disease. *International Journal Biochemistry & Cell Biology, 37*(2), 289-305.

Unnati, S., Ripal, S., Sanjeev, A., & Niyati, A. (2013). Novel anticancer agents from plant sources. *Chines Journal of Natural Medicines, 11*(1), 0016-0023.

Urrego, D., Tomczak, A. P., Zahed, F., Stuhmer, W., & Pardo, L. A. (2014). Potassium channels in cell cycle and cell proliferation. *Philosophical Transactions Royal Society of London. Series B Biological sciences, 369*(1638), 20130094.

Uttara, B., Singh, A. V., Zamboni, P., & Mahajan, R. T. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology, 7*(1), 65-74.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal Biochemistry & Cell Biology, 39*(1), 44-84.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40.

van den Heuvel, S., & Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. *Science, 262*(5142), 2050-2054.

van Muiswinkel, F. L., & Kuiperij, H. B. (2005). The Nrf2-ARE Signalling pathway: promising drug target to combat oxidative stress in neurodegenerative disorders. *Current Drug Targets CNS Neurological Disorders, 4*(3), 267-281.

Vane, J. R., Bakhle, Y. S., & Botting, R. M. (1998). Cyclooxygenases 1 and 2. *Annual Review Pharmacology and Toxicolology, 38*, 97-120.

Vanneman, M., & Dranoff, G. (2012). Combining immunotherapy and targeted therapies in cancer treatment. *Nature Reviews Cancer, 12*(4), 237-251.

Verma, S., & Singh, S. (2008). Current and future status of herbal medicines. *Veterinary World, 1*(11), 347-350.

Vermes, I., Haanen, C., Steffens-Nakken, H., & Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunology Methods, 184*(1), 39-51.

Viallard, J. F., Lacombe, F., Belloc, F., Pellegrin, J. L., & Reiffers, J. (2001). [Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology]. *Cancer Radiotherapie, 5*(2), 109-129.

Virolle, T., Adamson, E. D., Baron, V., Birle, D., Mercola, D., Mustelin, T., & de Belle, I. (2001). The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling. *Nature Cell Biology, 3*(12), 1124-1128.

Visconti, R., Della Monica, R., & Grieco, D. (2016). Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword. *Journal of Experimental Clinical Cancer Research, 35*(1), 153.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature, 408*(6810), 307-310.

Volkmann, N., Marassi, F. M., Newmeyer, D. D., & Hanein, D. (2014). The rheostat in the membrane: BCL-2 family proteins and apoptosis. *Cell Death and Differentiation, 21*(2), 206-215.

von Bernhardi, R. (2007). Glial cell dysregulation: a new perspective on Alzheimer disease. *Neurotoxicity Research, 12*(4), 215-232.

Vousden, K. H., & Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. *Cell, 137*(3), 413-431.

Wada, T., & Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene, 23*(16), 2838-2849.

Wagner, E. F., & Nebreda, A. R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nature Reviews Cancer, 9*(8), 537-549.

Wakabayashi, N., Slocum, S. L., Skoko, J. J., Shin, S., & Kensler, T. W. (2010). When NRF2 talks, who's listening? *Antioxidant & Redox Signaling, 13*(11), 1649-1663.

Walczak, H., & Krammer, P. H. (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Experimental Cell Research, 256*(1), 58-66.

Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., & Kerr, D. (2009). Genetic prognostic and predictive markers in colorectal cancer. *Nature Reviews Cancer, 9*(7), 489-499.

Wan, F., Anderson, D. E., Barnitz, R. A., Snow, A., Bidere, N., Zheng, L., . . . Lenardo, M. J. (2007). Ribosomal protein S3: a KH domain subunit in NF-kappaB complexes that mediates selective gene regulation. *Cell, 131*(5), 927-939.

Wang, J., Ding, N., Li, Y., Cheng, H., Wang, D., Yang, Q., . . . Fang, X. (2015). Insulin-like growth factor binding protein 5 (IGFBP5) functions as a tumor suppressor in human melanoma cells. *Oncotarget, 6*(24), 20636-20649.

Wang, J., Wang, Z., Wang, H., Zhao, J., & Zhang, Z. (2011). Curcumin Induces Apoptosis in EJ Bladder Cancer Cells via Modulating C-Myc and PI3K/Akt Signaling Pathway. *World Journal of Oncology, 2*(3), 113-122.

Wang, J., Yuan, L., Xiao, H., Xiao, C., Wang, Y., & Liu, X. (2013). Momordin Ic induces HepG2 cell apoptosis through MAPK and PI3K/Akt-mediated mitochondrial pathways. *Apoptosis, 18*(6), 751-765.

Wang, L., Jian, S., Nan, P., & Zhong, Y. (2004). Chemical composition of the essential oil of *Elephantopus scaber* from Southern China. *Zeitschrift fur Naturforschung C, 59*(5-6), 327-329.

Wang, P., Wu, P., Siegel, M. I., Egan, R. W., & Billah, M. M. (1995). Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *Journal of Biological Chemistry, 270*(16), 9558-9563.

Wang, W. W., Smith, D. L., & Zucker, S. D. (2004). Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. *Hepatology, 40*(2), 424-433.

Wang, W. Y., Tan, M. S., Yu, J. T., & Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Annals of Translational Medicine, 3*(10), 136.

Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Hunter, T., & Osada, H. (2004). M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. *Proceedings of National Academy of Sciences United States of America, 101*(13), 4419-4424.

Wee, Y. V. (2010). Inflammation in neurological disorders: a help or a hindrance? *Neuroscientist, 16*(4), 408-420.

Wesolowska, A., Nikiforuk, A., Michalska, K., Kisiel, W., & Chojnacka-Wojcik, E. (2006). Analgesic and sedative activities of lactucin and some lactucin-like guaianolides in mice. *Journal of Ethnopharmacology, 107*(2), 254-258.

Weydert, C. J., & Cullen, J. J. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nature Protocols, 5*(1), 51-66.

White, E., & DiPaola, R. S. (2009). The double-edged sword of autophagy modulation in cancer. *Clinical Cancer Research, 15*(17), 5308-5316.

White, R. L. (1998). Tumor suppressing pathways. *Cell, 92*(5), 591-592.

Whiteside, S. T., & Israel, A. (1997). I kappa B proteins: structure, function and regulation. *Seminars Cancer Biology, 8*(2), 75-82.

Williams, C. S., Mann, M., & DuBois, R. N. (1999). The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene, 18*(55), 7908-7916.

Williams, G. H., & Stoeber, K. (2012). The cell cycle and cancer. *Journal of Pathology, 226*(2), 352-364.

Wong, H. R., & Menendez, I. Y. (1999). Sesquiterpene lactones inhibit inducible nitric oxide synthase gene expression in cultured rat aortic smooth muscle cells. *Biochemical and Biophysical Research Communications, 262*(2), 375-380.

Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental Clinical Cancer Research, 30*, 87.

Wortzel, I., &Seger, R. (2011). The ERK Cascade: Distinct Functions within Various Subcellular Organelles. *Genes Cancer, 2*(3), 195-209.

Wu, J., Tang, Q., Zhao, S., Zheng, F., Wu, Y., Tang, G., & Hahn, S. S. (2014). Extracellular signal-regulated kinase signaling-mediated induction and interaction of FOXO3a and p53 contribute to the inhibition of nasopharyngeal carcinoma cell growth by curcumin. *International Journal of Oncology, 45*(1), 95-103.

Xu, G., Liang, Q., Gong, Z., Yu, W., He, S., & Xi, L. (2006). Antitumor activities of the four sesquiterpene lactones from *Elephantopus scaber* L. *Experimental Oncology, 28*(2), 106-109.

Yamagishi, H., Kuroda, H., Imai, Y., & Hiraishi, H. (2016). Molecular pathogenesis of sporadic colorectal cancers. *Chinese Journal of Cancer, 35*, 4.

Yamazaki, H., Tanji, K., Wakabayashi, K., Matsuura, S., & Itoh, K. (2015). Role of the Keap1/Nrf2 pathway in neurodegenerative diseases. *Pathology International, 65*(5), 210-219.

Yang, Z. J., Chee, C. E., Huang, S., & Sinicrope, F. A. (2011). The role of autophagy in cancer: therapeutic implications. *Molecular Cancer Therapeutics, 10*(9), 1533-1541.

Yeh, P. Y., Chuang, S. E., Yeh, K. H., Song, Y. C., Chang, L. L., & Cheng, A. L. (2004). Phosphorylation of p53 on Thr55 by ERK2 is necessary for doxorubicin-induced p53 activation and cell death. *Oncogene, 23*(20), 3580-3588.

Yeh, P. Y., Chuang, S. E., Yeh, K. H., Song, Y. C., Ea, C. K., & Cheng, A. L. (2002). Increase of the resistance of human cervical carcinoma cells to cisplatin by inhibition of the MEK to ERK signaling pathway partly via enhancement of anticancer drug-induced NF kappa B activation. *Biochem Pharmacol, 63*(8), 1423-1430.

Yet, S. F., Melo, L. G., Layne, M. D., & Perrella, M. A. (2002). Heme oxygenase 1 in regulation of inflammation and oxidative damage. *Methods in Enzymology, 353*, 163-176.

Yip, K. W., & Reed, J. C. (2008). Bcl-2 family proteins and cancer. *Oncogene, 27*(50), 6398-6406.

Yuste, J. E., Tarragon, E., Campuzano, C. M., & Ros-Bernal, F. (2015). Implications of glial nitric oxide in neurodegenerative diseases. *Frontiers in Cellular Neuroscience, 9*, 322.

Zhang, H. B. Kong, L. J., Hang, Q. L., Jiang, J. H., & Min, Z. D. (2011). Studies on triterpenes from E.scaber. *Chinese Journal of Experimental Traditional Medical Formulae, 3*, 101-103.

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *International Anesthesiology Clinics, 45*(2), 27-37.

Zhang, R., Humphreys, I., Sahu, R. P., Shi, Y., & Srivastava, S. K. (2008). *In vitro* and *in vivo* induction of apoptosis by capsaicin in pancreatic cancer cells is mediated

through ROS generation and mitochondrial death pathway. *Apoptosis, 13*(12), 1465-1478.

Zhang, S., Won, Y. K., Ong, C. N., & Shen, H. M. (2005). Anti-cancer potential of sesquiterpene lactones: bioactivity and molecular mechanisms. *Current Medicinal Chemistry Anticancer Agents, 5*(3), 239-249.

Zhang, S., & Yu, D. (2010). PI(3)king apart PTEN's role in cancer. *Clinical Cancer Research, 16*(17), 4325-4330.

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research, 12*(1), 9-18.

Zhong, L. M., Zong, Y., Sun, L., Guo, J. Z., Zhang, W., He, Y., . . . Lu, D. (2012). Resveratrol inhibits inflammatory responses via the mammalian target of rapamycin signaling pathway in cultured LPS-stimulated microglial cells. *PLOS ONE, 7*(2), e32195.

Zhu, Y., Choi, S. H., & Shah, K. (2015). Multifunctional receptor-targeting antibodies for cancer therapy. *Lancet Oncology, 16*(15), e543-554.

Zipp, F., & Aktas, O. (2006). The brain as a target of inflammation: common pathways link inflammatory and neurodegenerative diseases. *Trends in Neurosciences, 29*(9), 518-527.

Zoncu, R., Efeyan, A., & Sabatini, D. M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature Reviews Molecular Cell Biology, 12*(1), 21-35.

Zou, H., Yang, R., Hao, J., Wang, J., Sun, C., Fesik, S. W., . . . Armstrong, R. C. (2003). Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. *Journal of Biological Chemistry, 278*(10), 8091-8098.

Zuckerman, D. S., & Clark, J. W. (2008). Systemic therapy for metastatic colorectal cancer: current questions. *Cancer, 112*(9), 1879-1891.

Zwaal, R. F., Comfurius, P., & Bevers, E. M. (2005). Surface exposure of phosphatidylserine in pathological cells. *Cellular & Molecular Life Sciences, 62*(9), 971-988.

LIST OF PUBLICATIONS

I. Chan, C. K., Tan, L. T. H., Andy, S. N., Kamarudin, M. N. A., Goh, B. H., & Kadir, H. A. (2017). Anti-neuroinflammatory activity of *Elephantopus scaber* via activation of Nrf2/HO-1 signalling and inhibition of p38 MAPK pathway in LPS-induced microglia BV-2 cells. *Frontiers in Pharmacology*.

II. Chan, C. K., Chan, G., Awang, K., & Abdul Kadir, H. (2016). Deoxyelephantopin from *Elephantopus scaber* Inhibits HCT116 Human Colorectal Carcinoma Cell Growth through Apoptosis and Cell Cycle Arrest. *Molecules*, *21*(3), 385.

III. Chan, C. K., Supriady, H., Goh, B. H., & Kadir, H. A. (2015). *Elephantopus scaber* induces apoptosis through ROS-dependent mitochondrial signalling pathway in HCT116 human colorectal carcinoma cells. *Journal of Ethnopharmacology, 168*, 291-304.

PAPER PRESENTED

1. Chan, C. K., Tong, K. L., Wong, P. F. and Kadir, H. A. Deoxyelephantopin induced ROS-mediated autophagy and apoptosis via multiple signalling pathways in HCT116 human colorectal carcinoma *in vitro* and *in vivo* models. International Postgraduate Research Awards Seminar (InPRAS) 7th Mar-8th Mar 2016. (Oral presentation, International)
2. Chan, C. K., Tong, K. L., Wong, P. F. and Kadir, H. A. Deoxyelephantopin from Elephantopus scaber, a potential multi-target compound, activates apoptosis pathway and suppression of survival pathway on HCT116 human colon carcinoma cells. International Conference on Biomedical & Health Sciences Research 2015, Everly Hotel, Putrajaya, Malaysia, 25th-27th Jan 2015. (Oral presentation, International)
3. Chan, C. K. and Kadir, H, A. Elephantopus scaber activates both ROS-dependent intrinsic and extrinsic pathway on HCT116 human colon carcinoma cells. 19th Biological Sciences of Graduate Congress, National University of Singapore, Singapore, 12th-15th Dec 2014. (Oral presentation, International)
4. Chan, C. K. and Kadir, H, A. Elephant's foot (Elephantopus scaber) a potential therapeutic agents for colon cancer? International Student Congress of (bio)Medical Sciences (ISCOMS), University Medical Center Gronigen, Netherlands， 4thJune-7thJune 2013. . (Poster presentation, International)
5. Tang, L. Y., Chan, C. K. and Kadir, H, A. Induction of Apoptosis in HT-29 Human Colorectal Adenocarcinoma Cells by Geraniin, Poster presentation, 24th Intervasity Biochemistry Seminar 2013, Taylor's University Lakeside Campus, Subang Jaya, Selangor. (Best Poster Award, National)