# ANTI-CANCER EFFECTS OF 1’S-1’-ACETOXYCHAVICOL ACETATE AND ITS HEMI-SYNTHETIC ANALOGUES ON CANCER CELL LINES

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# ANTI-CANCER EFFECTS OF 1'S-1'-ACETOXYCHAVICOL ACETATE AND ITS HEMI-SYNTHETIC ANALOGUES ON CANCER CELL LINES

## ABSTRACT

1'S-1'-acetoxychavicol acetate (ACA) is a phenylpropanoid isolated from the rhizome of the wild ginger plant, *Alpinia conchigera* (Zingiberaceae). Nine analogues of ACA were hemi-synthesised and evaluated for their cytotoxic effects using MTT assay against breast, bladder, prostate, oral and liver human cancer cell lines. Only ACA and two analogues, 1'-acetoxyeugenol acetate (AEA) and 1'-acetoxy-3,5-dimethoxychavicol acetate (AMCA) showed significant cytotoxic effects. The aims of the research were to investigate if ACA and its two analogues, first, could exert anti-cancer effects via the proteasome and second, to explore the possible underlying mechanism of action as well as the structure-activity relationship (SAR) involving anti-proliferation, apoptosis induction and anti-migration effects in breast cancer cells. Since the ubiquitin-proteasome system (UPS) is seen as an effective system in modulating tumour cell proliferation, the proteasome-inhibitory potential of ACA, AEA and AMCA was investigated. Among the three different proteasome activities, the best inhibition by these three compounds was the chymotrypsin-like activity of 26S proteasome in MDA-MB-231 breast cancer cells. The docking analysis showed that 1'-acetoxy group is the key player of proteasome inhibition. However, the compounds were significantly less active compared to the commercial proteasome inhibitor, epoxomicin. This suggested that ACA and its analogues did not exert effective anti-cancer effects via the UPS system. However, ACA and its two analogues, AEA and AMCA inhibited the cell growth of MDA-MB-231 breast cancer cells significantly. The 1'- and 4-acetoxy, and methoxy group substituted at 3-position of the benzene ring were found to be important for anti-proliferation, whereas 4-hydroxy, methoxy group at 4- and 5-positions reduced the activity. Further investigation of these three compounds using DNA fragmentation assay showed that they markedly

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increased apoptosis of MDA-MB-231 cells. The expression levels of cleaved PARP, p53 and Bax were elevated whereas the expression of Bcl-2 and Bcl-xL were decreased after the treatment of ACA, AEA and AMCA. These findings suggested that ACA and the two analogues are able to inhibit MDA-MB-231 cell growth by inducing apoptosis via the mitochondrial apoptotic pathway. Also, the SAR between ACA and its analogues in anti-migration effects were analysed since ACA, AEA and AMCA effectively inhibited the migration of MDA-MB-231 cells. The structural requirements for anti-migration effects are the 1'- and 4-acetoxy, and 3-methoxy groups that were identified as essential for inhibition of the cancer cells migration. In contrast, the 4-hydroxy and 5-methoxy weaken the activity. The compounds also downregulated the expression level of pFAK/FAK, pAkt/Akt via the integrin β1-mediated signalling pathway. Collectively, ACA, AEA and AMCA are potentially beneficial anti-cancer agents by their ability to suppress growth, induce apoptosis and inhibit the migration of breast cancer cells.

**Keywords:** ACA hemi-synthetic analogues, SAR, apoptosis, anti-migration, ubiquitin-proteasome system

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**KESAN-KESAN ANTI-KANSER 1'S-1'-ASITOKSIKAVIKOL ASETAT DAN ANALOG-ANALOG HEMI-SINTETIKNYA PADA TITISAN-TITISAN SEL KANSER**

## ABSTRAK

1'S-1'-asitoksikavikol asetat (ACA) ialah suatu fenilpropanoid yang telah diasingkan daripada rizom tumbuhan halia hutan, *Alpinia conchigera* (Zingiberaceae). Sembilan analog ACA telah dihemi-sintesiskan dan dikaji bagi kesan sitotoksik mereka terhadap titisan-titisan sel kanser payudara, pundi air kencing, prostat, mulut dan hati manusia melalui ujian MTT. Hanya ACA dan dua analog, 1'-asitoksieugenol asetat (AEA) and 1'-asitoksi-3,5-dimetoksikavikol asetat (AMCA) menunjukkan kesan-kesan sitotoksik yang signifikan. Tujuan-tujuan penyelidikan ini adalah untuk mengkaji sama ada ACA dan dua analognya, pertama, boleh mengenakan kesan-kesan anti-kanser melalui proteasom dan yang kedua, untuk memeriksa mekanisme tindakan yang mungkin terbabit dan juga hubungan struktur-aktiviti (SAR) bagi kesan-kesan anti-proliferasi, induksi apoptosis, dan anti-migrasi pada sel-sel kanser payudara. Disebabkan sistem ubiquitin-proteasom (UPS) merupakan suatu sistem yang efektif dalam modulasi proliferasi sel barah, oleh itu potensi perencatan proteasom oleh ACA, AEA dan AMCA telah dikaji. Antara tiga aktiviti proteasom yang berbeza, perencatan yang terbaik oleh tiga kompaun ini adalah pada aktiviti seperti-kimotripsin 26S proteasom dalam sel MDA-MB-231. Analisis doking menunjukkan bahawa kumpulan 1'-asitoksi merupakan pemain utama dalam perencatan proteasom. Walaubagaimanapun, kompaun-kompaun ini adalah kurang aktif secara signifikannya jika berbanding dengan perencat proteasom komersial, epoxomicin. Ini menunjukkan ACA dan analog-analognya tidak memberi kesan-kesan anti-kanser yang efektif melalui sistem UPS. Walaubagaimanapun ACA dan dua analog, AEA dan AMCA merencatkan pertumbuhan sel-sel kanser payudara MDA-MB-231 secara signifikannya. Kumpulan 1'- dan 4-asitoksi, dan kumpulan metoksi yang disubstitusikan

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pada kedudukan 3 dalam gelang benzena, didapati adalah penting untuk anti-proliferasi, manakala 4-hidroksi, kumpulan metoksi pada kedudukan 4 dan 5 mengurangkan aktiviti tersebut. Penyelidikan selanjutnya terhadap tiga kompaun dengan ujian fragmentasi DNA menunjukkan bahawa mereka meningkatkan peratusan apoptosis sel-sel MDA-MB-231 secara ketaranya. Tahap ungkapan PARP terbelah, p53 dan Bax meningkat manakala Bcl-2 dan Bcl-xL menurun setelah rawatan ACA, AEA dan AMCA. Penemuan ini mencadangkan bahawa ACA dan dua analog boleh merencatkan pertumbuhan sel MDA-MB-231 dengan menginduksikan apoptosis melalui laluan apoptotik mitokondria. Selain itu, SAR antara ACA dan analognya dalam kesan-kesan anti-migrasi juga telah dianalisiskan kerana ACA, AEA dan AMCA merencatkan migrasi sel-sel MDA-MB-231 secara berkesannya. Keperluan struktur bagi kesan-kesan anti-migrasi yang telah dikenal pasti sebagai penting adalah kumpulan 1'- dan 4-asitoksi, dan 3-metoksi bagi perencatan migrasi sel kanser. Malahan, 4-hidroksi dan 5-metoksi melemahkan aktiviti ini. Kompaun-kompaun ini juga telah mengawal turun tahap ungkapan pFAK/FAK, pAkt/Akt melalui laluan syarat yang diperantarakan oleh integrin β1. Secara koletif, ACA, AEA dan AMCA berpotensi menjadi agen-agen anti-kanser yang bermanfaat dari kebolehan mereka untuk menyekat pertumbuhan, menginduksikan apoptosis dan merencatkan migrasi sel-sel kanser payudara.

**Kata kunci:** Analog-analog hemi-sintetik ACA, SAR, apoptosis, anti-migrasi, sistem ubiquitin-proteasom

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| Å | : | Ångströms |
| α | : | Alpha |
| β | : | Beta |
| °C | : | Degrees Celsius |
| µ | : | Micro |
| µg | : | Micrograms |
| µg/l | : | Micrograms per litre |
| µg/ml | : | Micrograms per millilitre |
| µl | : | Microlitre |
| µM | : | Micromolar |
| µm | : | Micrometre |
| ≤ | : | Less than or equal to |
| > | : | More than |
| % | : | Percentage |
| ® | : | Registered |
| (v/v) | : | Volume per volume |
| (w/v) | : | Weight per volume |
| 1D | : | One-dimensional |
| 2D | : | Two-dimensional |
| A | : | Absorbance |
| ABEs | : | Aspirin-based benzyl esters |
| $AC_2O$ | : | Acetic anhydride |
| ACA | : | 1’S-1’-acetoxychavicol acetate |
| ACS | : | American Cancer Society |

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| | | |
|---|---|---|
| AEA | : | 1'S-1'-acetoxyeugenol acetate / 1'-acetoxyeugenol acetate |
| Akt | : | Protein kinase B |
| AMCA | : | 1'-acetoxy-3,5-dimethoxychavicol acetate |
| AMP | : | Adenosine monophosphate |
| ANOVA | : | Analysis of variance |
| Apaf-1 | : | Apoptotic protease activating factor 1 |
| AP-1 | : | Activating protein-1 |
| APS | : | Ammonium persulfate |
| AR | : | Androgen receptor |
| ATCC | : | American Type Culture Collection |
| ATP | : | Adenosine Triphosphate |
| Bad | : | Bcl-2-associated death promoter |
| Bak | : | Bcl-2 homologous antagonist/killer |
| Bax | : | Bcl-2 associated X |
| BCA | : | Bicinchoninic acid |
| Bcl-2 | : | B-cell lymphocyte 2 |
| Bcl-w | : | Bcl-2-like protein 2 |
| Bcl-$X_L$ | : | B-cell lymphoma-extra large |
| BH | : | Bcl-2 homology |
| Bid | : | BH3 interacting-domain death agonist |
| Bik | : | Bcl-2 interacting killer |
| Bim | : | Bcl-2-like protein 11 |
| Bmf | : | Bcl-2-modifying factor |
| bp | : | Base pair |
| BSA | : | Bovine serum albumin |
| CARD | : | Caspase recruitments domain |

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| | | |
|---|---|---|
| CARIF | : | Cancer Research Initiative Foundation |
| Caspase | : | Cysteine aspartate protease |
| CC | : | Column chromatography |
| CD24 | : | Cluster of differentiation 24 |
| CD44 | : | Cluster of differentiation 44 |
| Cdc42 | : | Cell division control protein 42 homologue |
| $CDCl_3$ | : | Deuterated chloroform |
| CER I | : | Cytoplasmic Extraction Reagent I |
| CER II | : | Cytoplasmic Extraction Reagent II |
| $CH_2Cl_2$ | : | Dichloromethane |
| $CH_3COO$ | : | Acetoxy |
| $C_6H_{14}$ | : | Hexane |
| CKI | : | Cyclin-dependent kinase inhibitor |
| cm | : | Centimetre |
| c-Myc | : | Cellular myelocytomatosis viral oncogene homologue |
| $CO_2$ | : | Carbon dioxide |
| COSY | : | Correlation spectroscopy |
| COX-2 | : | Cyclooxygenase-2 |
| CR | : | Cytokine receptor |
| CSN | : | Constitutive photomorphogenesis 9 signalosome |
| CT | : | Chymotrypsin |
| C-Terminal | : | Carboxyl terminal |
| DD | : | Death domain |
| DEPT | : | Distortionless enhancement by polarisation transfer |
| $dH_2O$ | : | Distilled water |
| DISC | : | Death-inducing signalling complex |

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| | | |
|---|---|---|
| DMAP | : | 4-Dimethylaminopyridine |
| DMEM | : | Dulbecco's Modified Eagles Medium |
| DMSO | : | Dimethyl sulfoxide |
| DNA | : | Deoxyribonucleic acid |
| DNase | : | Deoxyribonuclease |
| dpf | : | Docking parameter file |
| DR | : | Death receptors |
| E2F | : | E2 transcription factor |
| EBV | : | Epstein-Barr virus |
| ECL | : | Enhanced chemiluminescence |
| ECM | : | Extracellular matrix |
| EDTA | : | Ethylenediaminetetraacetic acid |
| EGFR | : | Epidermal growth factor receptor |
| Egr-1 | : | Early growth response protein 1 |
| EMT | : | Epithelial to mesenchymal transition |
| ER | : | Endoplasmic reticulum |
| ER | : | Oestrogen receptor |
| FADD | : | Fas-associated death domain |
| FAK | : | Focal adhesion kinase |
| Fas | : | First apoptosis signal receptor |
| FBS | : | Foetal bovine serum |
| FDA | : | Food and Drug Administration |
| *g* | : | Gravity |
| G | : | Grams |
| GA | : | Genetic algorithm |
| GAPDH | : | Glyceraldehyde 3-phosphate dehydrogenase |

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| | | |
|---|---|---|
| GFRKs | : | Growth factor receptor kinases |
| GPCR | : | G protein-coupled receptors |
| gpf | : | Grid parameter file |
| GSH | : | Glutathione |
| GTPase | : | Guanosine triphosphatase |
| HBV | : | Hepatitis B virus |
| HCl | : | Hydrochloric acid |
| HCC | : | Hepatocellular carcinoma |
| HCV | : | Hepatitis C virus |
| HEPES | : | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HER-2 | : | Human epidermal growth factor receptor 2 |
| HMBC | : | Heteronuclear multiple-bond connectivity |
| HMEC | : | Human mammary epithelial cell |
| HPV | : | Human papillomavirus |
| H-ras | : | Harvey rat sarcoma viral oncogene homologue |
| HRP | : | Horse radish peroxidase |
| Hr | : | Hour |
| Hrs | : | Hours |
| HSC-4 | : | Human squamous carcinoma-variant 4 |
| HSQC | : | Heteronuclear single-quantum coherence spectroscopy |
| $IC_{20}$ | : | 20.0% inhibitory concentration |
| $IC_{50}$ | : | 50.0% inhibitory concentration |
| ID | : | Identity |
| IgE | : | Immunoglobulin E |
| IGF-1 | : | Insulin-like growth factor 1 |
| IGF-1/2 | : | Insulin-like growth factor 1/2 |

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| | | |
|---|---|---|
| IGF-IR | : | Insulin-like growth factor I receptors |
| IκB | : | Inhibitor of nuclear factor kappa B |
| IκB-α | : | Inhibitor of nuclear factor kappa B alpha |
| IKK | : | Inhibitor of nuclear factor kappa B kinase |
| IKKα/β | : | Inhibitor of nuclear factor kappa B alpha/beta |
| IL-1β | : | Interleukin 1 beta |
| IL-3 | : | Interleukin 3 |
| IL-4 | : | Interleukin 4 |
| IR | : | Infrared |
| kcal | : | Kilocalories |
| kDa | : | Kilodalton |
| Kg | : | Kilograms |
| LLC | : | Lilly Laboratories Cell |
| LLVY | : | Leucine-leucine-valine-tyrosine |
| LPS | : | Lipopolysaccharide |
| LRR | : | Leucine-arginine-arginine |
| M | : | Molar |
| mA | : | Milliampere |
| MCF-7 | : | Michigan Cancer Foundation-7 |
| MDa | : | Mega Dalton |
| MEGM | : | Mammary Epithelial Growth Medium |
| MeOH | : | Methanol |
| MET | : | Mesenchymal-epithelial transition |
| mg | : | Milligrams |
| mg/ml | : | Milligrams per millilitre |
| Mins | : | Minutes |

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ml : Millilitre

mM : Millimolar

mm : Millimetre

$mm^3$ : Millimetre cube

mmol : Millimole

MMP : Matrix metalloproteinase

MMP-2 : Matrix metalloproteinase-2

MMP-9 : Matrix metalloproteinase-9

MNCR : Malaysia National Cancer Registry

mol : Mole

MPT : Mitochondrial permeability transition

MS : Mass spectroscopy

mTOR2 : Mammalian target of rapamycin complex 2

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

NAD : Nicotinamide adenine dinucleotide

$NaSO_4$ : Sodium sulphate anhydrous

NCR : National Cancer Registry

NER : Nuclear Extraction Reagent

NF-κB : Nuclear factor kappa B

$NH_4Cl$ : Ammonium chloride

nLPnLD : Norleucine-proline-norleucine-aspartate

nm : Nanometre

NMR : Nuclear magnetic resonance

NOESY : Nuclear overhauser effect spectroscopy

Noxa : Phorbol-12-myristate-13-acetate-induced protein 1

N-terminal : Amino terminal

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| | | |
|---|---|---|
| $OCH_3$ | : | Methoxy |
| OH | : | Hydroxy |
| *p* | : | *p*-value of data statistical significance |
| p21 | : | Cyclin-dependent kinase inhibitor 1 |
| p27 | : | Cyclin-dependent kinase inhibitor 1B |
| p53 | : | Protein 53 |
| PAGE | : | Polyacrylamide gel electrophoresis |
| pAkt | : | Phosphorylated protein kinase B |
| PARP | : | Poly ADP ribose polymerase |
| PBS | : | Phosphate buffer saline |
| PCR | : | Polymerase chain reaction |
| PDB | : | Protein Data Bank |
| PDK1 | : | Phosphoinositide-dependent kinase 1 |
| PDK2 | : | Phosphoinositide-dependent kinase 2 |
| pFAK | : | Phosphorylated focal adhesion kinase |
| pH | : | Potential of hydrogen |
| PH | : | Pleckstrin homology |
| PI3K | : | Phosphatidylinositol 3-kinase |
| PIP3 | : | Phosphatidylinositol (3,4,5) trisphosphates |
| PR | : | Progesterone receptor |
| PSA | : | Prostate specific antigen |
| PSMA | : | Prostate-specific membrane antigen |
| pTEN | : | Phosphatase and tensin homologue |
| PUMA | : | p53 upregulated modulator of apoptosis |
| Rac1 | : | Ras-related C3 botulinum toxin substrate 1 |
| RANKL | : | Receptor activator of nuclear factor kappa B ligand |

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| | | |
|---|---|---|
| RBL-2H3 | : | Rat basophilic leukaemia |
| RelA/p65 | : | Transcription factor p65 |
| Rho | : | Rhodopsin |
| RLU | : | Relative light unit |
| rmstol | : | Root mean square deviation tolerance |
| RNA | : | Ribonucleic acid |
| rpm | : | Revolutions per minute |
| RPMI-1640 | : | Roswell Park Memorial Institute Medium |
| RT | : | Room temperature |
| SAR | : | Structure activity relationship |
| SD | : | Semi-dry |
| SDS | : | Sodium dodecyl sulphate |
| Secs | : | Seconds |
| SEM | : | Standard error mean |
| Ser15 | : | Serine 15 |
| SI | : | Selectivity index |
| Src | : | Steroid receptor coactivator |
| Suc | : | Succinyl |
| TAE | : | Tris base, acetic acid and EDTA |
| tBid | : | Truncated Bid |
| TBS | : | Tris-buffered saline |
| TBST | : | Tris-buffered saline with Tween-20 |
| TEMED | : | N,N,N',N'-Tetramethylethylenediamine |
| TGS | : | Tris-glycine-SDS |
| Thr1 | : | Threonine residue 1 |
| TIMP-1 | : | Tissue inhibitor of metalloproteinase 1 |

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| | | |
|---|---|---|
| TLC | : | Thin layer chromatography |
| ™ | : | Trademark |
| TNBC | : | Triple-negative breast cancer |
| TNF | : | Tumour necrosis factor |
| TNF-α | : | Tumour necrosis factor alpha |
| TRAIL | : | TNF-α-related apoptosis inducing ligand |
| TRADD | : | TNFR-associated death domain |
| Trp53 | : | Transformation-related protein 53 |
| U87MG | : | Uppsala 87 Malignant Glioma |
| Ub | : | Ubiquitin |
| U/ml | : | Units per millilitre |
| uPA | : | Urokinase plasminogen activator |
| UPS | : | Ubiquitin-proteasome system |
| USA | : | United States of America |
| UV | : | Ultraviolet |
| V | : | Volts |
| WHO | : | World Health Organization |
| X | : | Times |
| XO | : | Xanthine oxidase |
| ZEB | : | Zinc finger E-box binding homeobox |

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# CHAPTER 1: INTRODUCTION

Over the past 50 years, natural products presented impressive achievements in drug discovery (Mishra & Tiwari, 2011). Many plant-derived natural products became the vital source for discovery of anti-cancer drugs due to their structural diversity and participation in multiple anti-cancer mechanisms. However, major challenges have hindered the development of these natural products as pharmaceutical drugs. These include problems such as low production yields of natural products during scale-up efforts (Fett-Neto et al., 1992), inadequate natural resources (Datta & Srivastava, 1997) as well as their complex structures which impedes improved structural modifications and synthesis of compounds (Morrison & Hergenrother, 2014). Additionally, screening of numerous extracts and purified compounds from a variety of natural sources involves substantial expenditure and time. Due to these hindrances, it is crucial to perform structural modifications through organic hemi-synthesis to counter the problems.

1'S-1'-acetoxychavicol acetate (ACA) is a phenylpropanoid which can be found in the plant *Alpinia conchigera* (Zingiberaceae) (Awang et al., 2010). It is known to exhibit a broad range of biological properties such as anti-ulcer (Mitsui et al., 1976), anti-fungal (Janssen & Scheffer, 1985), inhibition of xanthine oxidase (XO) (Noro et al., 1988), inhibition of Epstein-Barr virus activation (Kondo et al., 1993) and anti-cancer activity (Murakami et al., 1996; Ohnishi et al., 1996; Tanaka et al., 1997b; Kobayashi et al., 1998). Moreover, it was reported that ACA and its natural analogue, AEA suppressed proliferation, induced apoptosis and reduced migration rate of various cancer cell lines *in vitro* as well as reduced tumour volume and side effects *in vivo* (Awang et al., 2010; Hasima et al., 2010; In et al., 2011; In et al., 2012). Due to the wide range of biological functions, hemi-synthesis of different ACA analogues was warranted for enhancement purposes (Murakami et al., 2000).

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Cancer, also known as malignant neoplasm, is a worldwide killer as it is a primary cause of death among many populations (Ferlay et al., 2013). Despite substantial strategies taken to combat cancer, it remains a fundamental burden to the poor and developing countries. In Malaysia it is a major health burden and ranked as the third fatal disease (Abdullah, 2016). Thus, development of effective anti-cancer drugs for cancer treatment is crucial.

The ubiquitin-proteasome system (UPS) plays an important role in regulating cellular processes such as apoptosis, angiogenesis, signal transduction, cell cycle and selective degradation of most intracellular proteins (Orlowski et al., 2003). The 26S proteasome is a multi-subunit protease complex made up of the 20S catalytic core and 19S regulatory particle. It is found in the nucleus and cytoplasm of eukaryotic cells (Peters et al., 1994). The 20S proteasome core is composed of two outer rings with seven α subunits (α1-α7) in each ring and two inner rings consisting of seven β subunits each. The 19S regulatory complex binds to the α subunits to induce the gate opening in the 20S proteasome. The β subunits with a terminal threonine residue play important roles in the proteolytic activities, such as chymotrypsin-like (β5), trypsin-like (β2) and peptidylglutamyl peptide hydrolysing-like (also known as caspase-like) (β1) activities (Seemuller et al., 1995). Proteins destined for degradation by the proteasome undergo polyubiquitination prior to their degradation. The proteasomal activity is seen to play a certain role in the progression of cancer in which some cancer-related proteins such as, p53, Bax, cyclins A, B, D and E, p27, IκB-α are targeted by proteasome (Ciechanover, 1998). Many proteasome inhibitors have been reported to exert anti-cancer effects on various cancer cells. For example, bortezomib, the first US Food and Drug Administration (FDA) approved proteasome inhibitor was found to kill cancer cells by regulating proteins associated with cancer survival (Kane et al., 2006). Thus, it is important to determine if the anti-cancer effects of a compound are mediated via the UPS.



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Apoptosis is a highly regulated cell death which is normally characterised by membrane blebbing, cell shrinkage, condensation of chromatin and DNA fragmentation followed by the rapid engulfment by macrophages (Renehan et al., 2001). In contrast to normal cells, cancer cells are able to evade the apoptosis process by their ability to disrupt the balance between anti-apoptotic and pro-apoptotic proteins (Juin et al., 2004; Vogler et al., 2009). Therefore, many natural and synthetic anti-cancer agents are vital to have the capability of inducing apoptosis in cancer cells.

Cancer metastasis is a complex, multi-step processes that involved tumour cells detaching, spreading and to grow at distant sites from the primary tumour site. The complex interaction between proteins from transmembrane receptors to transcription factors triggers multi-step cellular signalling events that leads to the cancer cell migration (Friedl & Brocker, 2000). The signalling events such as integrin-FAK-Src signalling pathway regulates the metastatic cells to loosen its extracellular matrix (ECM) adhesion (Hood & Cheresh, 2002). The epithelial to mesenchymal transition (EMT), another essential step in promoting cancer metastasis, allows the disruption of the cell-cell adhesion, matrix remodelling, increasing motility and invasiveness. These processes can be regulated by signalling pathways such as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Jing *et al.*, 2011). Thus, it is also crucial to search for anti-cancer agents that can modulate signalling pathways which consequently lead to anti-migration activities.

In this study, the effects of ACA and its hemi-synthetic analogues on the proliferation of various cancer cell lines were assessed. The involvement of UPS in the anti-cancer effects was also investigated. In addition, two other major anti-cancer properties, namely, apoptosis induction and anti-migration effects and their underlying molecular mechanisms were investigated. Therefore, the hemi-synthetic analogues of ACA would have improved anti-cancer properties with synthetically modified chemical structures,

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increased apoptosis induction and inhibition of migration effects. If these anti-cancer effects are regulated via the UPS, it would be more effective.

## 1.1 Study Objectives

i) To study the structure-activity relationship (SAR) between synthetically modified chemical structures of ACA and its analogues and their anti-cancer activity.

ii) To investigate the potential of ACA and its analogues to trigger apoptosis in cancer cells through regulation of proteasomal activity.

iii) To investigate the inhibitory effects of ACA and its analogues on purified and tumour-derived proteasome proteolytic activities.

iv) To identify molecular modes of binding between the proteasome and structural features of ACA and its analogues.

v) To analyse the levels of ubiquitinated proteins and proteasome target proteins after inhibition of the UPS.

vi) To determine the apoptotic protein regulation expression and metastasis-related pathways mediated by ACA and its analogues.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1 Natural Products

Over the past half-century, natural products have been widely used for the development of effective cancer chemotherapeutic agents (Mishra & Tiwari, 2011). The importance of natural products in cancer therapy was summarised in a report by (Newman & Cragg, 2016), where it was found that 83.0 % of FDA approved anti-cancer drugs from 1981 to 2014 were either natural products or synthetic products of natural compounds. The higher plant-derived compounds, such as paclitaxel, vincristine, vinblastine and bortezomib have been accepted as FDA drugs (Kane et al., 2003; Guérritte & Fahy, 2005; Cseke et al., 2006). Apart from these, some natural compounds such as the epipodophyllotoxin derivatives, maytansine, bruceantin, thalicarpine, camptothecin and lapachol have been examined through several epidemiological and experimental studies (Sieber et al., 1976). The natural compounds are advantageous with regard to appropriateness for oral intake, possession of multiple mechanisms of action and regulatory approval (Tsuda et al., 2004).

Vincristine is one of the vinca alkaloid anti-cancer drugs isolated from the leaves of field grown *Catharanthus roseus* in the Madagascar rain forests (Noble, 1990). Vincristine exhibited significant anti-tumour activity in patients with Hodgkin lymphoma and some forms of leukaemia (Devita et al., 1970). The compounds from the vinca alkaloid family have been discovered as potent inhibitors of cell proliferation and have been widely used in cancer therapy. The efficacy of vinca alkaloids against cancer cells by occupying the tubulin's building block structure (Bai et al., 1990), which in turn leads to cell arrest in mitosis (Gidding et al., 1999).

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Resveratrol (*trans*-3,5,4'-trihydroxystilbene) was first isolated in 1940 from the roots of white hellebore, *Veratrum grandiflorum* O. Loes but has later been found in grapes, berries and peanuts (Sarkar & Li, 2006). Several findings showed that resveratrol is capable to inhibit the proliferation of cancer cells including breast cancer (Mgbonyebi et al., 1998), oral squamous carcinoma (Elattar & Virji, 1999), prostate cancer (Hsieh & Wu, 1999), pancreatic cancer (Ding & Adrian, 2002), colon cancer (Delmas et al., 2002), ovarian carcinoma (Yang et al., 2003) and cervical carcinoma (Aggarwal et al., 2004).

Another important natural compound is curcumin (diferuloylmethane), a major component of the Indian spice turmeric, *Curcuma longa*, which has been described as an anti-inflammatory agent (Arora et al., 1971). It also has been the subject of intense study as anti-cancer molecules (Kawamori et al., 1999; Kim et al., 2009; Lai et al., 2011). The anti-cancer potential of curcumin is related to cell growth inhibition of various cancer cell types; downregulation of transcription factors NF-κB, AP-1 and Egr-1; reduced expression of COX-2, MMP-9, TNF-α and cyclin D1; inhibition of growth factor receptors EGFR and HER-2 and deactivation of several protein kinases involved in tumourigenesis (Aggarwal et al., 2003).

Apart from these, many other plant-derived natural products such as phenylpropanoids are regarded as fundamental source for discovery of anti-cancer drugs due to their structural diversity and broad array of anti-cancer activities.

### 2.1.1 Phenylpropanoids

Phenylpropanoids is the largest and most diverse group of secondary metabolites sourced from plants (Korkina, 2007). Phenylpropanoids can be found abundantly in human diet, spices, aromas, wines, essential oils and traditional medicine. These

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compounds are of great interest especially for medical use as anti-oxidant, anti-bacterial, anti-microbial and anti-cancer agents.

Eugenol is an important phenylpropanoid extracted from aromatic flower buds found in *Syzygium aromaticum*, which has been discovered to possess anti-inflammatory (Kim et al., 2003), anti-genotoxic (Han et al., 2007), anti-oxidant (Ito et al., 2005b) and anti-mutagenic (Miyazawa & Hisama, 2001) properties. Besides, it can induce apoptotic cell death in several cancer cells such as breast adenocarcinoma (Jaafari et al., 2012), colon carcinoma (Slameňová et al., 2009), prostate cancer (Ghosh et al., 2009) and oral squamous carcinoma (Carrasco et al., 2008). Eugenol has also significantly reduced the expression of Bcl-2, COX-2 and IL-1β in the HeLa cell line (Hussain et al., 2011). Moreover, eugenol treatment arrested the melanoma cells in the S phase of cell cycle, induced apoptosis and upregulated several enzymes involved in the base excision repair pathway, including E2F family members (Ghosh et al., 2005b). Pal and collaborators carried out *in vivo* analysis and showed that eugenol inhibited skin carcinogenesis in mice by downregulation of proliferation-associated genes c-*myc* and H-*ras* and anti-apoptotic gene *Bcl-2*, along with upregulation of pro-apoptotic genes *Bax*, *p53* and active caspase-3 (Pal et al., 2010). Hence, eugenol is a phenylpropanoid with notable anti-cancer effects.

Another kind of phenylpropanoid, myristicin, 1-allyl-3,4-methylenedioxy-5-methoxybenzene can be isolated from carrot, nutmeg and parsley (Hallstrom & Thuvander, 1997). Lee and collaborators reported that myristicin induced cytotoxicity on human neuroblastoma SK-N-SH cells by apoptotic mechanism via cleavage of PARP, accumulation of cytochrome c and activation of caspase-3 (Lee et al., 2005). In brief, myristicin shows great potential as an effective anti-cancer agent.

One of the natural products that can be extracted from fennel, star anise, dill, basil and tarragon is anethole (1-methoxy-4-(1-propenyl)benzene) (Nakagawa & Suzuki, 2003).



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Anethole induced cytotoxicity effects on various cancer cells such as fibroblastic sarcoma (Choo et al., 2011), cervical carcinoma (Stoichev et al., 1967) and hepatocytes (Marshall & Caldwell, 1992). Choo and friends showed that anethole inhibited proliferation, adhesion and invasion of highly metastatic human HT-1080 fibrosarcoma cells via inhibition of MMP-2 and MMP-9 and upregulation of MMP inhibitor TIMP-1 (Choo et al., 2011). Anethole also reduced tumour weight, tumour volume and body weight in Ehrlich ascites tumour-bearing mice (Al-Harbi et al., 1995). Anethole exhibits significant anti-cancer activities both *in vitro* and *in vivo*.

Hydroxychavicol, 1-allyl-3,4-dihydroxybenzene, is a phenolic compound present in Piper beetle leaf (Chakraborty et al., 2012). A study by Nakagawa and collaborators revealed that hydroxychavicol induced cytotoxic effects on rat hepatocytes (Nakagawa et al., 2009).

Overall, phenylpropanoids are important secondary metabolites that displayed strong anti-cancer therapeutic effects. Further studies of these natural compounds are required for the development of new drug candidates in cancer treatment.

### 2.1.2 *Alpinia conchigera* (Zingiberaceae)

*Alpinia conchigera* Griff. (Figure 2.1) is an herbaceous, perennial plant grown in shaded and moist environment of rainforest and valley, with the heights of up to 126 cm when fully matured (Burkill et al., 1966). This plant belongs to *Alpinia* genus, which is the largest and most common genus in the Zingiberaceae family with 230 species throughout tropical and subtropical Asia, especially in Bengal, Malaysian Peninsular and Sumatera (Holttum, 1950; Ibrahim et al., 2000; Kress et al., 2005). The Zingiberaceae, also known as the Ginger family is the largest family in the order of Zingiberales, with about 53 genera and over 1200 species (Kress et al., 2002). The order and classification

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of *Alpinia conchigera* within the taxonomic hierarchy of *Alpinia* genus has been conducted using DNA-based approaches and illustrated in Figure 2.2.

*Alpinia conchigera* is also known as *lengkuas ranting*, *lengkuas kecil*, *lengkuas padang*, *lengkuas geting* or *chengkenam* in Malaysia (Burkill et al., 1966; Janssen & Scheffer, 1985; Kress et al., 2005). This species is reported to be useful as traditional medicine, spice, food, condiment, dye and flavouring (Ibrahim et al., 2000). In some states of Peninsular Malaysia, the rhizome is used as condiment and the young shoots are used for vegetable dish. In terms of its medicinal uses, rhizome extract of *Alpinia conchigera* have been used by Malays as medicine to treat skin fungal infections and consumed as post-partum medicine (Ibrahim et al., 2009). People in Thailand use the rhizomes in traditional medicine to relieve gastrointestinal disorders and in the preparation of Thai food dishes (Athamaprasangsa et al., 1994).

The chemical constituents of *Alpinia conchigera* have been the subject of previous studies. The first, by Yu and friends, reported that the fruits of *Alpinia conchigera* contained compounds including nonacosane, β-sitosterol, 1'-acetoxychavicol acetate and 1'-acetoxyeugenol acetate, the two latter phenylpropanoid derivatives exhibiting anti-inflammatory activity (Yu, 1988). Later, Athamaprasangsa and collaborators identified chavicol, chavicol acetate, 1'-hydroxychavicol acetate, 4-acetoxycinnamyl alcohol and 4-acetoxycinnamyl acetate, together with six monoterpenoids, five diarylheptanoids and two flavonoids, which were obtained from the rhizomes and fruits of *Alpinia conchigera* in Thailand (Athamaprasangsa et al., 1994). However, no quantitative data were given. In 1995, another report about 34 essential oil components from the rhizomes of *Alpinia conchigera* from the southern region of Peninsular Malaysia, among which β-bisabolene, β- sesquiphellandrene and 1,8-cineole were found to be the major components (Sirat & Nordin, 1995). Besides, Wong and his team reported that the rhizome oil of *Alpinia*

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*conchigera* from the northern region of Peninsular Malaysia yielded 50 compounds with the majority being terpenoids (Wong et al., 2005). Another active compound, cardamomin (2',4'-dihydroxy-6'-methoxychalcone) isolated from *Alpinia conchigera* was recognised as an inhibitor of NF-κB activation, which suppressed LPS-induced degradation, phosphorylation of IκB-α and the RelA/p65 subunit of NF-κB (Lee et al., 2006).

**Figure 2.1:** *Alpinia conchigera* Griff.

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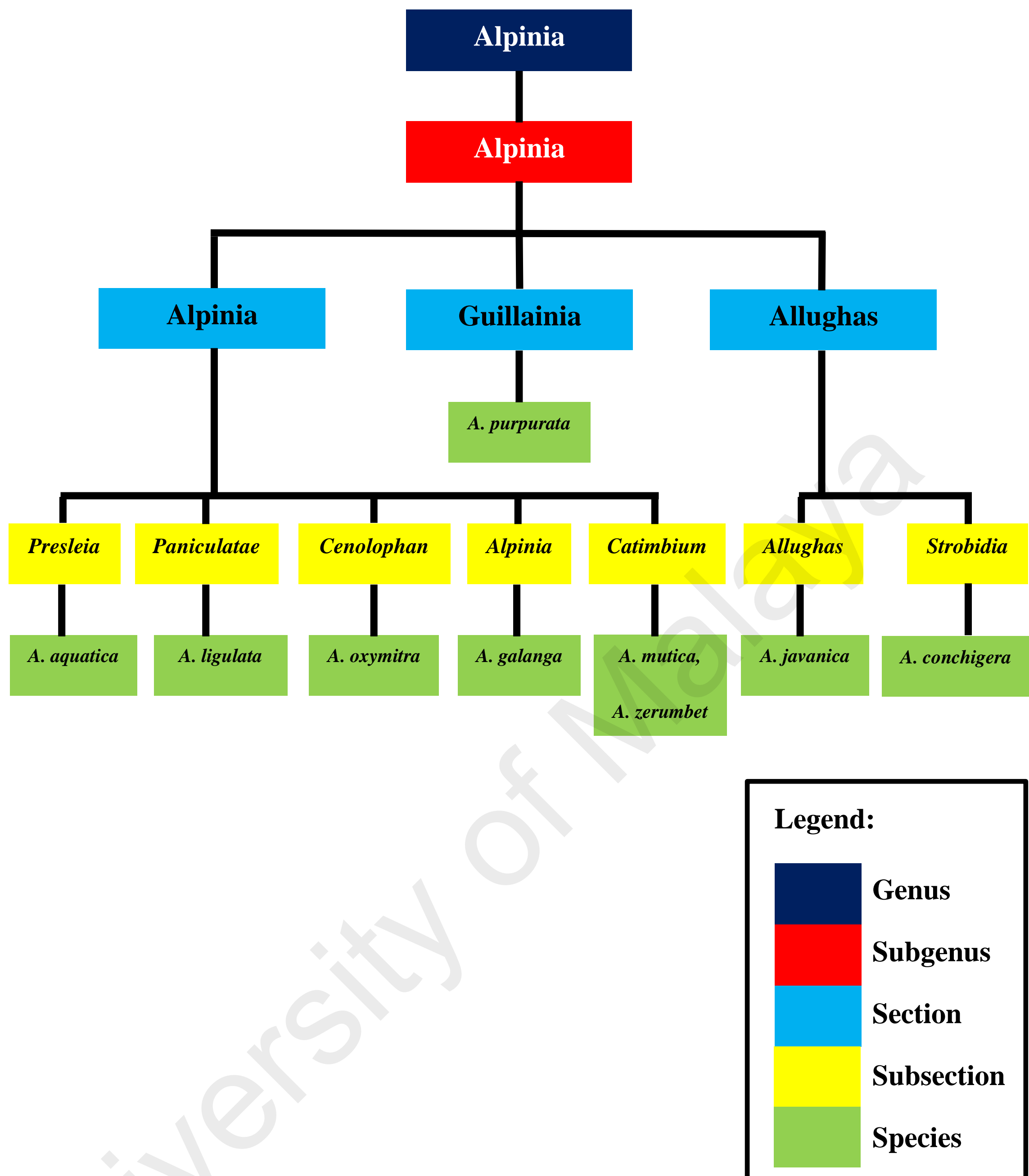


**Figure 2.2:** The order and classification of the *Alpinia* species up to section and subsection levels (Reproduced with permission from Smith, 1990).

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### 2.1.3 1'S-1'-Acetoxychavicol Acetate (ACA)

1'S-1'-acetoxychavicol acetate (ACA) is a naturally occurring compound found in many ginger species, which belongs to the phenylpropanoid group. The active chemical structure of ACA is illustrated in Figure 2.3. ACA has been reported to possess anti-ulcer (Mitsui et al., 1976), anti-fungal (Janssen & Scheffer, 1985), anti-tumourigenic (Itokawa et al., 1987), anti-inflammatory (Nakamura et al., 1998), anti-oxidative (Kubota et al., 2001) and anti-allergic (Matsuda et al., 2003a) properties.

Until recently, studies on ACA presented its *in vitro* inhibitory effects on cancers, such as Ehrlich ascites tumour cells (Moffatt et al., 2000), myeloid leukaemia (Ito et al., 2004), human T cell lymphoma (Ichikawa et al., 2005), breast carcinoma (Campbell et al., 2007) and human colorectal cancer (Baradwaj et al., 2017).

*In vivo* studies have also depicted that ACA has potent cancer chemopreventive effects on chemically induced tumour formation in mouse skin (Murakami et al., 1996), rat oral (Ohnishi et al., 1996), rat colon (Tanaka et al., 1997a), rat oesophagus (Kawabata et al., 2000) and Syrian hamster pancreas (Miyauchi et al., 2000).

In terms of anti-cancer mechanism, ACA was shown to induce apoptosis in Ehrlich ascites tumour cells through modulation of polyamine metabolism and caspase-3 activation (Moffatt et al., 2000). It also was found to exert anti-proliferative effects on myeloma cells *in vitro* and *in vivo* through induction of apoptosis via mitochondrial- and Fas-mediated dual mechanism (Ito et al., 2004). Further studies showed that ACA inhibited the activation of NF-κB (Ito et al., 2005a) by preventing the IκB-α kinase activity (Ichikawa et al., 2005) and by blocking the RANKL-induced NF-κB activation (Ichikawa et al., 2006). More recently, ACA isolated from rhizomes of the *Alpinia conchigera* Griff. was reported to suppress proliferation, induce apoptosis and reduce

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migration rate on various cancer cell lines *in vitro* as well as to reduce tumour volume and side effects *in vivo* (Awang et al., 2010; In et al., 2012). ACA was also shown to inhibit the constitutive activation of NF-κB through suppression of its kinase, IKKα/β. Despite all these reports revealing ACA mechanisms, the involvement of the ubiquitin-proteasome system (UPS) in mediating its anti-cancer effects is unknown.

In 2000, the structure-activity relationships (SAR) of ACA on its anti-cancer activity were analysed based on the inhibitory activity of ACA analogues on EBV activation (Murakami et al., 2000). According to this study, it was found that 2'-3' terminal double bond of ACA was highly important for its biological activity. Moreover, it was also concluded that the acetoxy group in ACA was crucial in cellular permeability properties because analogues without 1'-acetoxy group resulted in the reduction of activity. Based on the use of esterase inhibitor tests in Raji cells, it was also suggested that acetoxy group attached at ACA was subjected to acetate elimination via hydrolysation by intracellular esterases in order to maintain its retention within the cells, thus resulting in an intracellular modified ACA candidate structure which targets specific downstream molecules (Murakami et al., 2000). An overall summary on the structural factors of ACA regulating the anti-cancer activity based on Murakami's study is illustrated in Figure 2.4. Another report on SAR of ACA depicted that substitution of acetoxy group with acetamide group markedly decreased the inhibitory activities of ACA analogues on HL-60 leukaemia cells (Misawa et al., 2015). Even though the SAR of ACA on certain anti-cancer activity has been studied, its structure-activity relationships on anti-proliferation and anti-migration effects have yet to be identified.

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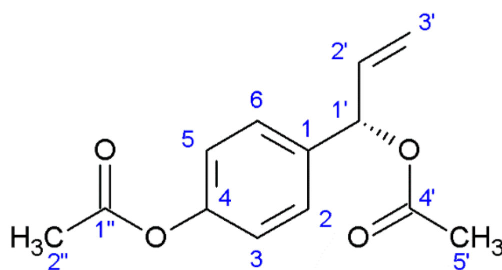


**Figure 2.3:** Chemical structure of 1'S-1'-acetoxychavicol acetate (ACA).

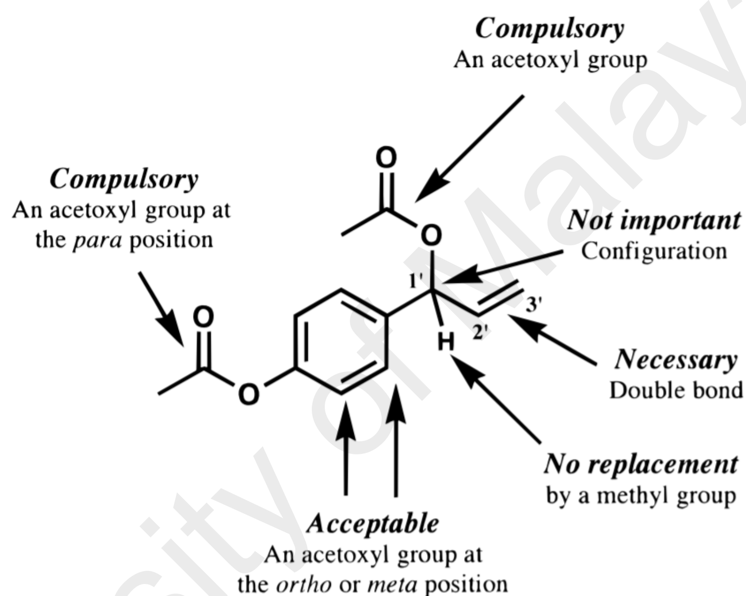


**Figure 2.4:** Summary of important structural factors of ACA for evaluation as anti-cancer inhibitor of EBV activation (Reproduced with permission from Murakami et al., 2000).

### 2.1.4 1'S-1'-Acetoxyeugenol Acetate (AEA)

1'S-1'-acetoxyeugenol acetate (AEA) is a closely related analogue of ACA, which can be found naturally in various wild gingers of Zingiberaceae family. AEA has an active chemical structure similar to ACA, but differing from ACA in respect to the additional methoxy group attached at 3' position of the benzene ring, as illustrated in Figure 2.5. Studies by Matsuda and collaborators have reported AEA isolated from *Alpinia galanga* induced inhibition on the ethanol-induced gastric mucosal lesions in rats (Matsuda et al.,

2003b) and exerted strong anti-allergic effects via inhibition of ear passive cutaneous anaphylaxis reactions in mice and the antigen-IgE-mediated TNF-α and IL-4 production, both of which involve in the late phase of type I allergic reactions in RBL-2H3 cells (Matsuda et al., 2003a).

The anti-cancer properties of AEA isolated from *Alpinia conchigera* showed higher cytotoxicity potency than ACA, inhibited cell cycle progression and induced apoptotic effects via dysregulation of NF-κB pathway on MCF-7 breast cancer cells (Hasima et al., 2010; In et al., 2011; In et al., 2012).

Despite numerous reports on AEA activity and structure from different ginger species, there have not been any studies conducted on the anti-cancer effects of synthetic AEA related to apoptotic induction via modulation of UPS and anti-migration effects.

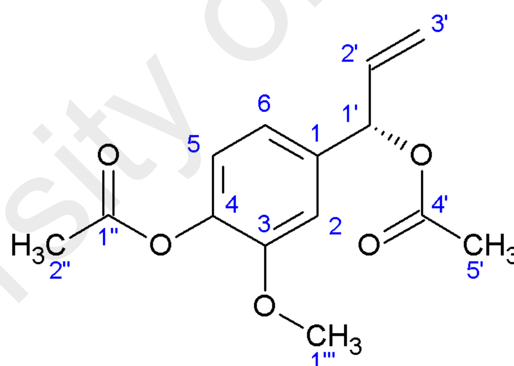


**Figure 2.5:** Chemical structure of 1’S-1’-acetoxyeugenol acetate (AEA).

### 2.1.5 Limitations on The Application of Plant Natural Products

Humans have been using plant-derived natural products as medicines for about 1000 years. Despite the intensive discovery of natural compounds as anti-cancer drugs, it is reported that only 5 to 15% of higher plants have been systematically evaluated for the presence of biologically active compounds (Kinghorn & Balandrin, 1993). In other



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words, many challenges have hindered the development of higher plant-derived natural products as pharmaceutical drugs. The secondary metabolites isolated from the plants typically possess highly complex structures which impedes the synthesis of these compounds on an industrial scale (Morrison & Hergenrother, 2014). Additionally, screening of numerous extracts and purified compounds from a variety of sources involves substantial expenditure and time. For example, although a total synthesis procedure was established for the key anti-cancer drug, paclitaxel by Holton and Nicolaou, but industrial production of paclitaxel via this method was not commercially viable (Holton et al., 1994; Nicolaou et al., 1994).

A significant outcome of the use of plants for medical purposes is of the sudden depletion of wild populations of these plants. For example, wild mountain ginseng (*Panax ginseng*) in Korea is highly appreciated for its medical properties, largely mediated by ginsenosides (Leung & Wong, 2010). Consequently, the populations of wild *P. ginseng* reduced massively and thus a single plant might sell for many thousands of dollars. In a similar case, paclitaxel extracted from the endangered and slow growing Pacific Yew (*Taxus brevifolia*) was inadequate to meet the projected demands (El & Diallo, 2000). In brief, low production yields of natural products during scale-up efforts (Fett-Neto et al., 1992) and inadequate natural resources (Datta & Srivastava, 1997) become the major problems in the development of natural compounds as anti-cancer drugs. Due to these limitations, it is imperative to perform structural modifications on potential compounds through organic hemi-synthesis, as this would allow discovery of analogues with higher efficacy.

## 2.2 Cancer

Cancer is a class of diseases in which abnormal cells multiply autonomously and uncontrollably. The occurrence of cancer can be in any tissue or organ of the body, so

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there are many diverse cancer types, which can vary significantly in their behaviour and response to treatment. A mass of tumour cells forms malignant cancers, which are capable to invade adjacent and distant body sites, leading to the destruction of the normal tissues. These cells can also spread throughout the body via blood and lymphatic system, in a process called metastasis. As shown in Figure 2.6, the malignant growth typically is governed by the cellular physiological alterations such as autonomous proliferation, inattention to growth inhibitory signals, suppression of apoptosis, immortalisation and stimulation of angiogenesis, invasion and metastasis (Hanahan & Weinberg, 2011). Based on these transformations, malignant cells are functionally different from the normal cells. This is due to the dysregulation of signalling pathways that control fundamental cellular processes such as, cell growth, apoptosis and migration (Kreeger & Lauffenburger, 2010). Once the perturbations of the pathways have occurred, cancer cells are able to develop in the absence of normal restrictions.

Cancer is one of the non-communicable diseases that upset the world, accounting for 8.8 million deaths in 2015 and is the world's second biggest killer (WHO, 2017). There were 14.1 million cases of cancer diagnosed around the world in 2012 (Ferlay et al., 2013) and it is expected to escalate about twofold within the next two decades, with approximately 21.6 million new cancer cases in the year 2030 (ACS, 2017). As a result of the growth of aging populations, it was estimated that about 13.0 million cancer patients will die from different types of cancer in 2030. In Malaysia, cancer is the third common cause for death, after cardiovascular and respiratory diseases (Abdullah, 2016). As reported by the Malaysia National Cancer Registry (MNCR), there were 103,407 cancer incidences in the country during the period of 2007 to 2011, of which 45.2% of the patients were males and 54.8% were females (Azizah et al., 2016). The top five common cancers among Malaysian males were colorectal (16.3%), lung (15.8%), nasopharyngeal (8.1%), lymphoma (6.8%) and prostate (6.7%) cancer. Meanwhile,

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cancers of the breast (32.1%), colorectal (10.7%), cervix uteri (7.7%), ovary (6.1%) and lung (5.6%) were the five most common cancers in Malaysian females.

The formation of cancer can be multifactorial and results from a complex interaction between genetic and environment. The contribution of genetic predispositions such as, inherited mutation and hormones towards cancer risk is only 5-10% of all cancer, whereas the remaining 90-95% are associated with environmental and lifestyle factors (Anand et al., 2008). Lifestyle factors are considered to play an important role in cancer etiology such as, high body mass index, unhealthy diet with low fruit and vegetable consumption, physical inactivity, drinking of alcohol and tobacco use (WHO, 2017). Tobacco is one of the documented chemical carcinogen responsible for increased risk of cancer and death throughout the world, accounting for 22% of all cancer deaths (Forouzanfar et al., 2016). In addition to lifestyle related factors, radiation and environmental pollutants also contributed to the transformation of normal cells to cancerous cells (Belpomme et al., 2007). Some known carcinogenic infections involving human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV) and Epstein Barr virus (EBV) are risk factors for cancer and mostly affecting people in low and middle-income countries (Plummer et al., 2016).

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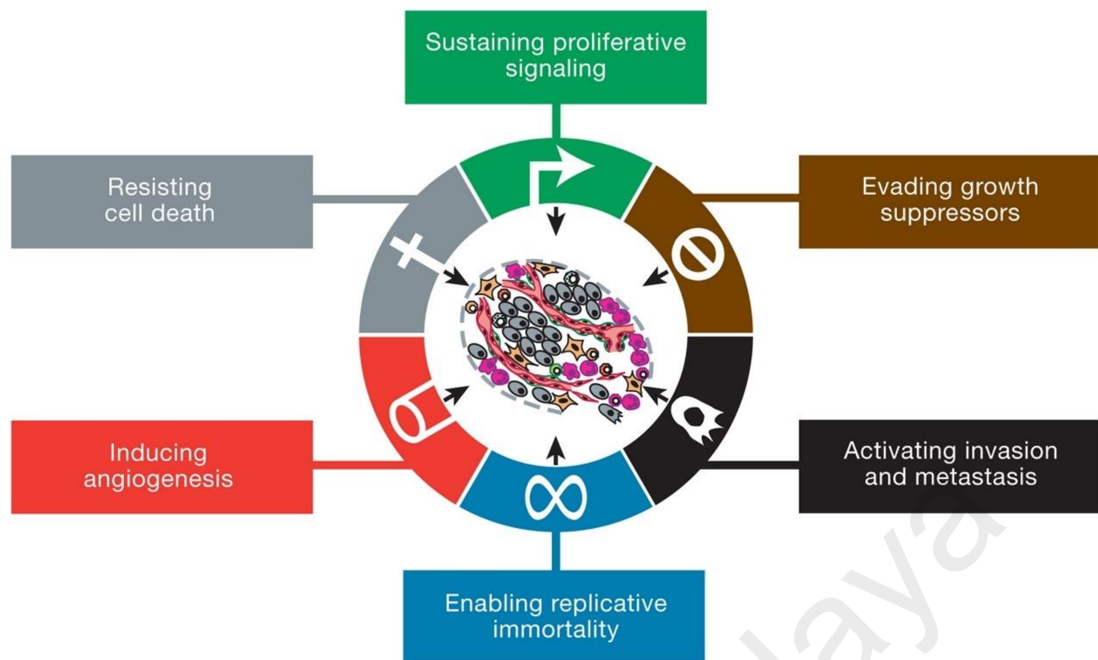


**Figure 2.6:** The hallmarks of cancer (Reproduced with permission from Hanahan & Weinberg, 2011).

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### 2.2.1 Breast Cancer

Breast cancer is a heterogeneous group of diseases originating from the epithelial cells lining the lobules and terminal ducts. According to the International Agency for Research on Cancer, breast cancer was the second most common cancer in the world with approximately 1.7 million new cases diagnosed in 2012 (Ferlay et al., 2013). It also ranked as the fifth most frequent type of cancer that increases the mortality rate in women. In 2017, the estimated number of new cases and deaths due to breast cancer in the United States was 255,180 and 41,070 respectively (ACS, 2017). In Malaysia, the National Cancer Registry (NCR) reported that there were 18,343 breast cancer cases amounting to 17.7% of total cancer cases in 2007-2011, making it the most common malignancy among Malaysia (Azizah et al., 2016). The established risk factors related to breast cancer are hormone exposure, family genotypes, alcohol consumption, early menarche, late menopause, low parity and post-menopausal obesity (Kolonel et al., 2004).

In this study, the MDA-MB-231 and MCF-7 cells are well-characterized cell lines and therefore are useful as *in vitro* models of breast cancer. MDA-MB-231 cell line was isolated from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma (Cailleau et al., 1978). It is highly invasive and known as triple-negative breast cancer (TNBC) cell line as it lacks oestrogen receptor (ER) expression, progesterone receptor (PR) expression and HER-2 (human epidermal growth factor receptor 2) amplification (Liu et al., 2003; Chavez et al., 2010). Similar to other invasive cancer cell lines, the invasiveness of the MDA-MB-231 cells is controlled by the proteolytic degradation of the extracellular matrix (Tryggvason et al., 1987). The presence of unique features such as, downregulated claudin-3 and claudinin-4 and the low expression of proliferation marker Ki67, has led MDA-MB-231 to be classified as 'claudin-low' subtype (Prat et al., 2010; Willmann et al., 2015). MDA-MB-231 cells exhibit the $CD44^{+}/CD24^{-/low}$ antigenic profile (Sheridan et al., 2006) and display epithelial-mesenchymal transition (EMT) features such as, reduced E-cadherin levels (Mbalaviele et al., 1996).

MCF-7 is a human breast adenocarcinoma cell line that was established from the pleural effusion of the mammary glands from a 69-year-old Caucasian woman in 1970 (Soule et al., 1973). Contrary to MDA-MB-231 cell line, epithelial-like MCF-7 cell line is oestrogen receptor positive cell line which has been the most popular models for breast cancer chemoprevention studies thus far (Kern et al., 1994; Punglia et al., 2005). In addition, MCF-7 was found to be non-invasive, expresses relatively high levels of insulin-like growth factor I receptors (IGF-IR) (Dickson et al., 1986), shows positive expression of E-cadherin (Hiraguri et al., 1998), possesses epidermal growth factor receptors (Biscardi et al., 1998) and progesterone receptors (Sutherland et al., 1988), and contains low amounts of endogenous caveolin (Paterson et al., 2003). Thus, it has been classified as 'luminal A' subtype (Holliday & Speirs, 2011). Besides, it retains several common

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properties of differentiated mammary epithelium such as, the ability of the cells to process oestradiol via oestrogen receptors in cytoplasm and the capability to form domes.

In brief, MDA-MB-231 breast cancer cells exhibit enhanced invasive properties compared to MCF-7 cells. Thus, MDA-MB-231 cell line is suitable for the study of anti-migration effects.

### 2.2.2 Bladder Cancer

Bladder cancer generally originates from the inner lining of the bladder, which is called the urothelium. The most common type of bladder cancer is urothelial carcinoma. There are also different variants of bladder cancer such as squamous cell carcinoma, small-cell carcinoma and adenocarcinoma. Globally, bladder cancer is the ninth most commonly diagnosed cancer, with an estimated 430,000 new cases in 2012 (Ferlay et al., 2013). About three-quarters of all bladder cancer patients were men. In Malaysia, bladder is the ninth most frequent cancer type among males and the twenty-sixth most common cancer among females. In 2007-2011, a total of 1,877 cases of bladder cancer were registered with NCR in Malaysia with 1,477 being males and 400 females (Azizah et al., 2016). Tobacco smoking has been identified as the main risk factor for bladder cancer (Burger et al., 2013; Ng et al., 2014). Besides, infection with Schistosoma haematobium is the major cause in parts of Northern and sub-Saharan Africa (Parkin, 2006).

RT-112 and EJ-28 were used to represent bladder cancer in this study. RT-112 is a type of human bladder carcinoma cell line that derived from a woman with untreated primary urinary bladder carcinoma in 1973 (Steele et al., 1983). It is a well-differentiated cell line that reproducibly grows as stratified epithelium (Fujiyama et al., 2001) and are found to be tumourigenic in the nude mice model (Marshall et al., 1977). This cell line displays positive expression of E-cadherin and hence it loss do not have invasive property

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(Gaetje et al., 1995; Bindels et al., 2000). This type of cell line has been used as model of superficial non-invasive tumours (Fujiyama et al., 2001). On the other hand, EJ-28 cell line is isolated from a patient with anaplastic carcinoma (Hastings & Franks, 1983). This poorly differentiated cell line lacks of E-cadherin and thus is considered as a metastatic cancer cell line (Fujiyama et al., 2001). In addition, EJ-28 cells were not tumourigenic in the nude mice (Marshall et al., 1977).

### 2.2.3 Prostate Cancer

Prostate cancer begins when cells in the prostate gland start to proliferate uncontrollably. Normal prostatic epithelium contains luminal cells, basal cells and a small component of neuroendocrine cells that scattered in prostatic glands (Sun et al., 2009). The most common type of prostate cancer is the adenocarcinoma which featured the absence of basal-like cells, luminal differentiation including glandular formation, the expression of androgen receptor (AR) and prostate-specific antigen (PSA) (Tai et al., 2011). Notably, minor case of prostatic adenocarcinoma contains small population of neuroendocrine tumour cells (Huang et al., 2007).

Worldwide, prostate cancer is the second most common cancer type in men and fourth most common cancer type among populations. It was estimated that 1.1 million men were diagnosed with prostate cancer and 307,000 deaths in the year 2012 (Ferlay et al., 2013). In Malaysia, prostate cancer is the sixth most common cancer among male where 1,163 cases of prostate cancer were reported by NCR in year 2007-2011 (Azizah et al., 2016). The risk factors for prostate cancer are age, race/ethnicity and family history (Gann, 2002). Dietary is also an important risk factor for prostate cancer (Yim et al., 2005).

Despite the availability of multiple approaches to treat prostate cancer, there is no effective therapy for the treatment of androgen-independent stage of prostate cancer

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which often arises after hormonal deprivation or ablation therapy (Feldman & Feldman, 2001). Moreover, the high recurrence of apoptosis resistance hormone refractory prostate cancer indicated that chemotherapy or radiotherapy were unable to improve the patient's condition (Koivisto et al., 1998).

The cancer cell line used to represent prostate cancer in this study was PC-3 cell line. It is a commonly used cell line, derived from advanced androgen independent bone metastasis prostate cancer in a 62-year-old Caucasian (Kaighn et al., 1979). This cell line has a minimised dependence upon serum for growth when compared to normal prostatic epithelial cells and does not respond to androgens, glucocorticoids, or epidermal or fibroblast growth factors. PC-3 cells have low testerone-5-alpha reductase and acidic phosphatase activity, do not express PSA and are PSMA-negative (prostate-specific membrane antigen) (Ghosh et al., 2005a).

### 2.2.4 Oral Cancer

Oral cancer is one of the few oral diseases encountered by the dental team that can cause significant morbidity and premature mortality despite advances in treatments such as chemotherapy, surgery, radiotherapy and combination therapy. The most common site for oral cancer is the tongue which occurs on the posterior lateral border and ventral surfaces of tongue, followed by the floor of the mouth, gingival, buccal mucosa, labial mucosa and hard plate (Tanaka & Ishigamori, 2011). The common risk factor for the development of oral cancer in Western countries is the consumption of tobacco (Warnakulasuriya et al., 2005) and alcohol (Ogden, 2005), whereas in Asian countries, the use of smokeless tobacco products such as betel quid and gutkha is the leading cause of oral cancer (Jeng et al., 2001; Boffetta et al., 2008).

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In the United States alone, oral cancer accounts for about 2% or 32,670 of all reported cancer cases, and contributes to approximately 1% or 6,650 of total cancer deaths (ACS, 2017). In Malaysia, the NCR reported that in 2007-2011, oral cancer cases were generally uncommon, and there were 1,299 cases amounting to 2.9% of all cancer cases and 1,191 cases amounting to 2.1% of all cancer cases among males and females respectively (Azizah et al., 2016).

In order to maximise the effects of killing the cancer cells while minimise the damage of normal tissue, many modern approaches have been adapted for the treatment of oral cancer. Currently, there are six common chemotherapeutic drugs including cisplatin, paclitaxel, docetaxel, methotrexate and bleomycin used to treat oral cancer. These drugs have a considerable level of undesirable side effects. Thus, it is important to search for better chemotherapeutic agents that can fight against oral cancer with minimal side effects.

To represent oral cancer in this study, HSC-4 cell line was used. It is a human oral cancer cell derived from a 64-year old Japanese male diagnosed with squamous carcinoma of the tongue. It was first established in Tokyo Medical and Dental University by Momose and collaborators. HSC-4 cells have an epithelial-like morphology and are devoid of viral DNA. They were found to express low amounts of COX-2 (Momose et al., 1989). COX-2 is an inflammatory enzyme that helps to catalyse the conversion of arachidonic acid to the prostaglandin E and is correlated with various cancer phenotypes (McAdam et al., 1999).

### 2.2.5 Liver Cancer

The liver is made up mainly of cells called hepatocytes. Hepatocellular carcinoma (HCC) is a primary liver cancer that generally starts in hepatocytes. In the year 2012, it

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was reported that liver cancer is the fifth most common cancer, accounting for 9.1% of all cancer cell deaths worldwide (Ferlay et al., 2013). In 2017, the estimated number of new cases and deaths from liver and intra-hepatic bile duct cancer in the US are 40,710 and 28,920 respectively (ACS, 2017). In Malaysia, liver cancer incidence was ranked sixth among males making up 6.7% of all cancer cases reported, and twelfth among females corresponding to 1.9% of all reported cancer cases (Azizah et al., 2016). The important risk factors for the development of HCC are viral hepatitis (HBV and HCV) (Tomimatsu et al., 1993), excessive alcohol intake (Batey et al., 1992), dietary aflatoxins (Liu & Wu, 2010) and hemochromatosis (Ko et al., 2007).

HepG2 is a human liver hepatocellular carcinoma cell line isolated from a 15-year old Caucasian male. It has an epithelial-like morphology and has been widely used as cellular reference model in pharmaceutical studies. HepG2 cells were reported to produce a variety of proteins such as, complement (C4), C3 activator, fibrinogen, alpha-fetoprotein and prothrombin (Knowles & Aden, 1983). They also express different kinds of liver-specific metabolic functions, including those linked to the cholesterol and triglyceride metabolism, thus making them an important *in vitro* model system (Javitt, 1990). At present, this cell line is actively used for cytotoxic evaluations (Koschutnig et al., 2009).

## 2.3 Ubiquitin-Proteasome System (UPS)

The ubiquitin-proteasome system (UPS) is the major pathway to mediate intracellular protein degradation. Substrate recognition and degradation within the UPS is highly controlled and therefore ensures a specific regulation of protein levels in numerous cellular processes such as, apoptosis, angiogenesis, signal transduction and cell cycle (Orlowski & Dees, 2003). The fundamental protease within the ubiquitin-proteasome system is the proteasome, a multicatalytic enzyme complex of about 2.5 MDa (Finley, 2009; Ciechanover, 2013).

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### 2.3.1 Proteasome

The 26S proteasome is a large multi-subunit protease complex located both in the nucleus and cytoplasm of eukaryotic cells (Peters et al., 1994). This complex is composed of a 20S catalytic core and 19S regulatory particle (Arrigo et al., 1988; Ganoth et al., 1988) as shown in Figure 2.7.

The 20S proteasome is made up of two outer α and two inner β subunits with seven distinct subunits per ring (Groll et al., 1997). The proteasome possesses highly unique secure arrangement for the specific degradation to prevent occurrence of uncontrolled proteolysis of cellular proteins. The active sites are safely situated inside the 20S catalytic core particle, normally found in a closed state with only a narrow entry pore. Hereby, the outer α-rings block the way to the proteolytic chamber in its closed conformation. Therefore, the 19S regulatory complex will bind to the α subunits to induce the gate opening and activation of 20S catalytic particles in an ATP-dependent manner (Groll et al., 2000; Finley, 2009; Silva et al., 2012).

The β subunits with a terminal threonine residue play a role in the proteolytic activities and cleave proteins into oligomeric peptides of 3-28 amino acids length. There are at least three major peptidase activities associated with the catalytic β subunits: chymotrypsin-like (β5), trypsin-like (β2) and peptidylglutamyl peptide hydrolysing-like (also known as caspase-like) (β1) activities (Seemuller et al., 1995). The β5 subunit prefers cleavage after hydrophobic residues, β2 cleaves after basic residues and β1 subunit cleaves on the C-terminal side of acidic residues (Borissenko & Groll, 2007).

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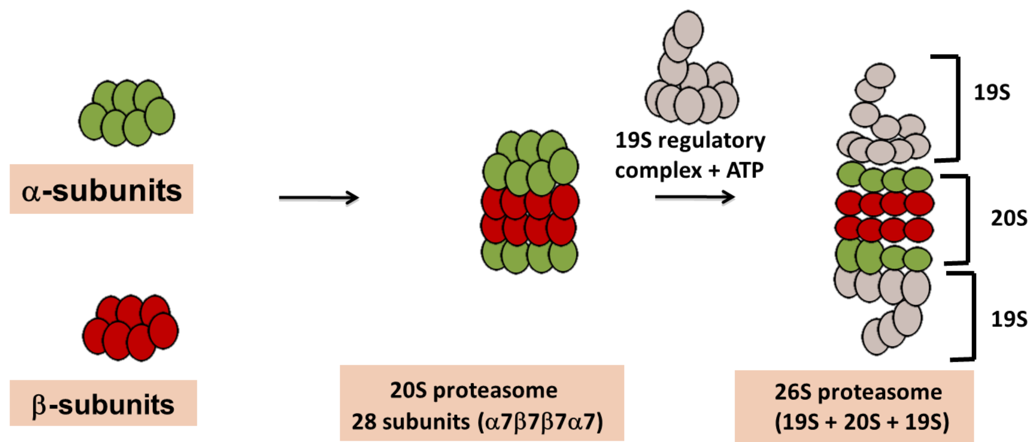


**Figure 2.7:** Composition of 26S proteasome (Reproduced with permission from Hasima & Aggarwal, 2014).

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### 2.3.2 Protein Degradation by UPS

The ubiquitin-proteasome system is responsible for the degradation of about 90% of all intracellular proteins, thereby serving as the main protein destruction machinery of the cell (Meiners et al., 2014). For a given protein to be aimed for degradation, the protein undergoes polyubiquitination and degradation by proteasome. Ubiquitination is a common degradation signal for the UPS and involves a cascade of E1 ubiquitin-activating, E2 ubiquitin-conjugating and E3 ubiquitin ligase enzymes. In this cascade, E1 (plus ATP) first adenylates the carboxy-terminal carboxylate of ubiquitin (Ub), forming Ub–AMP, and then forms a Ub thioester intermediate (E1–Ub). Ubiquitin is transferred from E1 to E2, and then to the protein target with the ligation assistance from E3. E2 enzymes promote further linkage of ubiquitin molecules, thereby creating a polyubiquitin chain on the protein (Ciechanover, 2015). Protein tagged with ubiquitin chains is recognised by the non-ATPase subunits Rpn10 and Rpn13 of the 19S regulatory particle. The deubiquitinase Rpn11 then cleaves off the ubiquitin chain in an ATP dependent manner and the protein substrate is unfolded and carried into the catalytic core of 20S proteasome

to undergo degradation into oligomeric peptides (Matyskiela et al., 2013). The overview of polyubiquitination and degradation of protein is depicted in Figure 2.8.

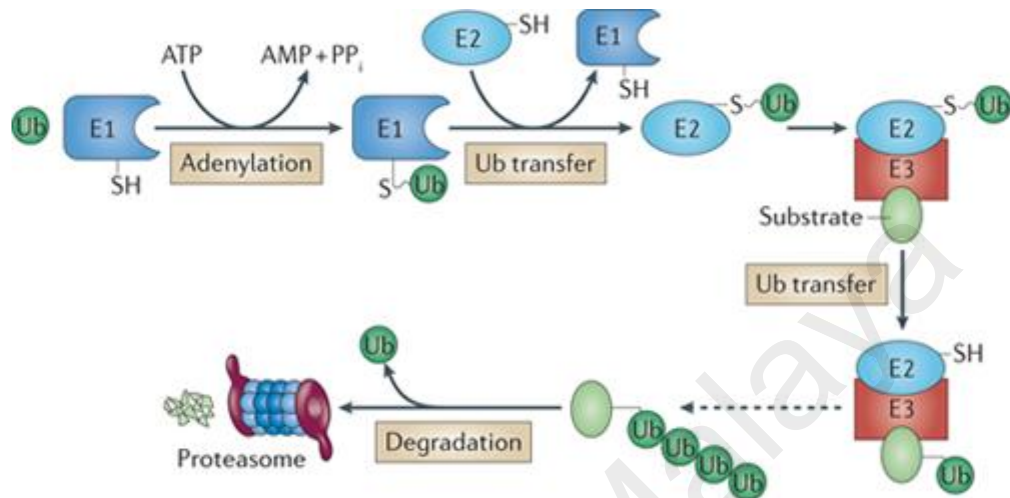


**Figure 2.8:** Degradation of a protein via the ubiquitin/proteasome pathway (Reproduced with permission from Maupin-Furlow, 2011).

### 2.3.3 Role of UPS in Cancer

The proteasomal activity plays a part in the pathogenesis of many diseases, including cancer, in which some regulatory proteins are either lost via acceleration of degradation or stabilised via deceleration of degradation (Ciechanover, 1998). Various proteins associated with cancer survival have been found to be targeted by the proteasome, including tumour suppressor protein p53 (Blagosklonny, 2002), pro-apoptotic protein Bax (Li & Dou, 2000), cyclins A, B, D and E (Glotzer et al., 1991; Won & Reed, 1996; Diehl et al., 1997; Chen et al., 2004), cyclin-dependent kinase inhibitor (CDKI) p27 (Pagano et al., 1995; Sun et al., 2001) and the inhibitor of NF-κB, IκB-α (Perkins, 2000). Furthermore, proteasome inhibition has been shown to activate several apoptotic pathways via accumulation of pro-apoptotic factors and downregulation of anti-apoptotic mediators. Indeed, inhibition of the proteolytic function of the 26S proteasome has been

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shown to enhance apoptosis in a number of cancer cells (Frankland-Searby & Bhaumik, 2012). Moreover, inhibition of the proteasome in various cancer cells prevents activation of NF-κB leading to downregulation of genes related to angiogenesis, survival and growth while apoptosis is upregulated (Hideshima et al., 2011). Figure 2.9 shows the effects of proteasome inhibition on different cancer pathways, which consequently contribute to cancer prevention.

Bortezomib is the first US Food and Drug Administration (FDA) approved proteasome inhibitor (Kane et al., 2006). It has been reported to possess anti-cancer effects on various cancer cell lines and animal xenografts models (Adams, 2002; Kondagunta et al., 2004; Jagannath et al., 2005). The proteasomal inhibition by bortezomib involves multiple mechanisms of action such as, stabilisation of cell-cycle regulatory proteins p21 (Adams et al., 1999), inhibition of nuclear factor kappa B (NF-κB) activation (Palombella et al., 1994) and suppression of anti-apoptotic proteins Bcl-2 (Qin et al., 2005).

Although bortezomib is an effective proteasome inhibitor, toxicity is a major obstacle in treatment. Therefore, there is a need to search for other proteasome inhibitors with less or minimal toxic side effects. Curcumin is one natural compound shown to mediate its cytotoxicity via the proteasome which is being investigated. Curcumin is a natural compound derived from the yellow curry spice turmeric. It has been found to module the UPS through several pathways including suppression of the protease activities of the proteasome, with the CT-like mechanism being the most prominent (Milacic et al., 2008), inhibition of COP9 signalosome (CSN), an important regulator of the UPS (Henke et al., 1999), and suppression of ubiquitin isopeptidases, a family of deubiquitinases that rescue ubiquitin for its reuse by the 26S proteasome system (Mullally & Fitzpatrick, 2002).

Curcumin could either upregulate or downregulate its target proteins through inhibition of proteasome or activation of proteasome. Some of the target proteins which

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are upregulated by curcumin through inhibition of proteasome: inhibition of degradation of IκB-α that leads to inhibition of NF-κB activation (Singh & Aggarwal, 1995), inhibition of p53 that leads to stabilisation of the protein in tumour cells and induces apoptosis (Jana et al., 2004), and induction of cytotoxicity by accumulation of ubiquitinated proteins and cyclin B (O'Sullivan-Coyne et al., 2009). On the other hand, proteasome activation by curcumin leads to downregulation of some proteins such cyclin D1 in prostate and breast cancer cells (Mukhopadhyay et al., 2002), cyclin D1 and cyclin E in prostate cancer cells (Srivastava & Singh, 2004), COX-2 in HeLa cervical cells (Neuss et al., 2007), Bcl-2 in lung cancer cells (Chanvorachote et al., 2009).

From the study with curcumin, it clearly showed that the modulation of proteasome activity could lead to upregulation and downregulation of large numbers of proteins that have been closely linked with cancer cell survival and proliferation. Thus, it is crucial to study on how compounds could induce anti-cancer effects on cancer cells via the UPS.

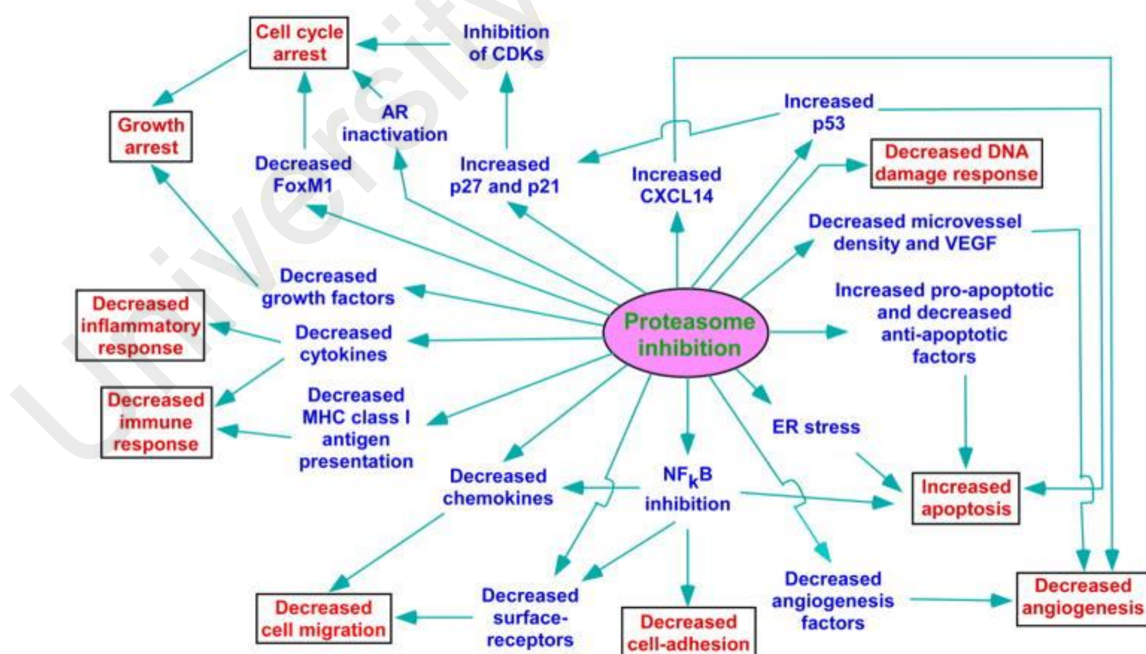


**Figure 2.9:** Implications of the proteasome inhibition on different pathways for cancer prevention (Reproduced with permission from Frankland-Searby & Bhaumik, 2012).

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## 2.4 Apoptosis

Apoptosis was defined as physiological death with distinctly different pattern of cell death from necrosis (Kerr et al., 1972). In ancient Greek, apoptosis means 'falling off' of petals from flowers or leaves from tree (Sankari et al., 2015). During apoptosis, the undesired cells will undergo several biochemical changes that could lead to the cellular morphological changes and ultimately to cell death. Apoptosis plays an important role in the early development and growth of tissues, and maintenance of cellular homeostasis (Norbury & Hickson, 2001). In addition, apoptosis also plays a crucial role as a defence mechanism against certain pathological conditions such as cancer (Del Bello et al., 2001; Choi, 2006). It has been shown that evasion of apoptosis can be seen in cancer cells and thus the induction of apoptosis is a highly desirable therapeutic strategy in cancer.

From the morphological point of view, cells undergoing apoptosis generally encounter cell shrinkage, chromatin condensation, cytoplasmic blebbing as well as formation of apoptotic bodies which are finally engulfed by macrophages and parenchymal cells (Kerr et al., 1972). Besides, irregularities in cellular shape also occur due to the cleavage of several cytoskeleton components by caspases. On the other hand, one of the major biochemical hallmark of apoptosis is the formation of DNA degradation by DNases that excise the internucleosomal regions into double-stranded DNA of 180-200 base pairs (Wyllie et al., 1980).

Apoptosis is a highly coordinated and often ATP-dependent process that involves complex cascade of molecular events. Thus far, there are three apoptotic pathways which have been identified: one is the extrinsic or death receptor-mediated pathway, the second is the intrinsic or mitochondrial-mediated pathway and lastly the perforin/granzyme pathway (Elmore, 2007). As illustrated in Figure 2.10, although these pathways are initiated differently, but they are linked and converge on the same execution pathway

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involving caspase-3 and eventually leads to apoptosis (Igney & Krammer, 2002; Elmore, 2007).

In the extrinsic pathway, caspase activation is mediated by death receptors (DR) which belong to the tumour necrosis factor (TNF) receptor gene superfamily that bind to TNF-α-related apoptosis inducing ligand (TRAIL) and are activated through ligation (Locksley et al., 2001). These DRs share similar cysteine-rich extracellular domains as well as the conserved cytoplasmic domain called the 'death domain' (Ashkenazi & Dixit, 1999). This death domain is able to transmit the death signal from the cell surface by binding with similar regions on cytoplasmic adaptor molecules such as, the Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD) through a death domain-death domain interaction (DD-DD) (Ashkenazi & Dixit, 1999; Hengartner, 2000; Strasser et al., 2000). Binding of FADD and TRADD would then form the death-inducing signalling complex (DISC) together with procaspase-8, followed by the catalytic processing and subsequent release of active caspase-8. The presence of activated caspase-8 then triggers the execution pathway involving caspase-3 to promote apoptosis (Kischkel et al., 1995).

In the intrinsic pathway, apoptosis induced by intracellular signals that may act positively such as, radiation, toxins, hypoxia, viral infections and free radicals or negatively in the absence of growth factors, cytokines and hormones (Elmore, 2007). These stimuli would lead to changes in mitochondrial permeability transition (MPT), causing the loss of mitochondrial membrane potential and provoking the release of several polypeptides from the mitochondria. One of this is the important cytochrome c which is an electron transport chain protein. Cytochrome c would accumulate in the cytoplasm and binds with the apoptotic protease-activating factor-1 (Apaf-1) causing the oligomerisation of Apaf-1 in the presence of ATP to form a complex called the

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apoptosome. Apaf-1 then directly binds to initiator caspases, procaspase-9 through homotypic interaction involving its caspase recruitments domain (CARD) (Budihardjo et al., 1999; Garrido et al., 2006). The complex activates procaspase-9 into caspase-9, which will then lead to activation of the execution pathway (Kaufmann & Hengartner, 2001).

On the other hand, the perforin/granzyme pathway is utilised by cytotoxic T-cells in mediating cytotoxicity. Activation of the pathway is by the release of the transmembrane pore-forming molecule perforin, with a subsequent exophytic release of cytoplasmic granules containing granzyme A or granzyme B through the pore and into the target cell (Trapani & Smyth, 2002). The granzyme A pathway induces apoptosis via activation of caspase-independent cell death via single-stranded DNA damage (Martinvalet et al., 2005) while granzyme B induces killing by activating the mitochondrial pathway or direct activation of caspase-3 (Goping et al., 2003).

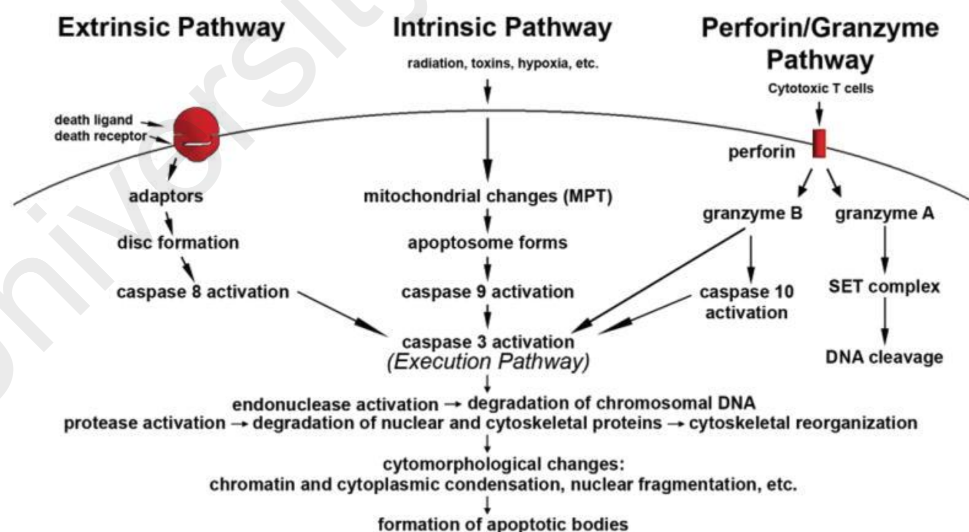


**Figure 2.10:** Illustration of extrinsic, intrinsic and perforin/granzyme pathways of apoptosis (Reproduced with permission from Elmore, 2007).

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### 2.4.1 Role of Apoptosis in Cancer

In the event of cancer, disruption of balance occurs between cell proliferation and apoptosis, and mutations in apoptotic pathways lead the cells with genetic abnormalities to continue their life. Over the years, many studies pointed out to the relationship between tumourigenesis and the attrition of apoptosis. The defects in apoptotic pathways resulting in acquired resistance towards apoptosis have been identified as a major hallmark in most, and perhaps all types of cancer (Hanahan & Weinberg, 2011).

The possibility that apoptosis serves as a barrier to cancer development was first proposed in 1972, when it was reported that the removal of hormone leads to substantial apoptosis in the fast growing, hormone-dependent tumour cells (Kerr et al., 1972). The discovery and recognition of the bcl-2 oncogene as having anti-apoptotic activity accelerated in the investigation of apoptosis at the molecular level (Vaux et al., 1988). Since then, various studies conducted on other oncogenic proteins such as, c-myc (McDonnell & Korsmeyer, 1991), IGF-1, Fas, Bcl-xL (Hueber et al., 1997) and many others, have further indicated the apoptotic program of cells can be triggered by an over-expression of oncogenes (Hanahan & Weinberg, 2011).

The development of cancer cells involves the evasion of apoptosis which allows the cells to proliferate infinitely. One of the methods to acquire the resistance to apoptosis by cancer cells is the mutation of p53 tumour suppressor gene as reported in 50% of cancers in humans (Harris, 1996). Another mechanism is the phenomenon of 'tumour counter attack' whereby tumour cells expressing Fas, which can counter attack and kill anti-tumour lymphocytes via apoptosis (O'Connell et al., 1999). It was also reported that Fas-death signalling is abrogated in a high fraction of lung can colon carcinoma cell lines (Pitti et al., 1998). Besides this, extracellular factors such as, IGF-1/2 and IL-3, intracellular signals from Ras and loss of pTEN expression can deliberate survival signals

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that resulted in the activation of PI3K-AKT pathway to transmit anti-apoptotic signals and enables cancer cells to evade apoptosis (Downward, 1998; Evan & Littlewood, 1998; Cantley & Neel, 1999).

The illustration of signalling circuit governing apoptosis has shown how apoptosis is triggered in response to different types of physiological stress in cells and this apoptotic circuit is generally constricted at some point in cancer cells, allowing them to progress to high-grade malignancy (Lowe et al., 2004; Adams & Cory, 2007). Thus, the understanding of this circuit allows for the development of multiple anti-cancer therapies which are intended to restore the abnormal apoptotic programs found in cancer cells or by inducing apoptosis through regulation in the expression of genes related to apoptosis.

### 2.4.2 Role of Bcl-2 Family Proteins in Apoptosis

The Bcl-2 family proteins are known as the important gatekeeper to the apoptotic cell death. This group of structurally related proteins have either pro- or anti-apoptotic properties that interact with one another. Bcl-2 was the first pro-survival protein in the family to be discovered (Reed, 2008). The *BCL-2* gene was discovered as the translocated locus (14:18) in tumour cells of follicular lymphoma patients (Tsujimoto et al., 1984). This chromosome translocation governs the deregulation of the normal Bcl-2 expression pattern, resulting in the formation of cancer (Tsujimoto et al., 1985; Nunez et al., 1989).

Following the discovery of Bcl-2, a large number of Bcl-2 related proteins have been isolated as depicted in Figure 2.11 (Cory & Adams, 2002). Based on the homology and functions of each protein, the Bcl-2 family is classified into the following three subfamilies: (i) A subfamily including Bcl-2, Bcl-xL and Bcl-w, all of which exert anti-apoptotic activity and share sequence homology in four domains, BH (Bcl-2 homology) 1 through BH4. (ii) A subfamily represented by Bax and Bak, which share sequence

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homology at BH1, BH2 and BH3 but not at BH4, although significant homology at BH4 is also found in some members. All these proteins exert pro-apoptotic activity. (iii) A subfamily including Bik and Bid, all of which are pro-apoptotic and share sequence homology only within BH3.

One of the eye-catching properties of Bcl-2 family proteins is their ability to form homodimers and heterodimers (Oltvai et al., 1993). Hetero-dimerisation between anti-apoptotic and pro-apoptotic members of this family is suggested to inhibit the biological activity of their partners (Oltvai et al., 1993; Yang et al., 1995), and is mediated through the insertion of BH3 domain of a pro-apoptotic protein into a hydrophobic groove formed by BH1, BH2 and BH3 domains of an anti-apoptotic protein (Sattler et al., 1997). In addition, some of these proteins have been indicated to regulate apoptosis independently of each other (Knudson & Korsmeyer, 1997). In either case, the ratio between anti-apoptotic and pro-apoptotic members of the Bcl-2 family helps determine, in part, the susceptibility of cells to a death signal. Thus, the Bcl-2 family of proteins acts as a vital life-death decision point within the common pathway of apoptosis.

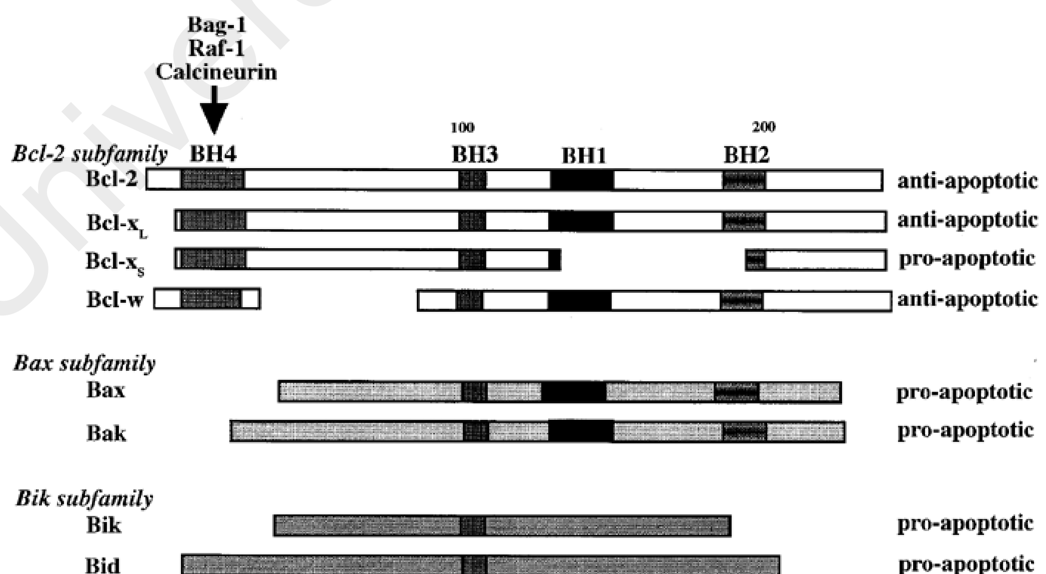


**Figure 2.11:** Homology of Bcl-2 family proteins. The Bcl-2 family can be categorised into three subfamilies: The Bcl-2 cohort promotes cell survival, whereas the Bax and Bik cohort facilitate apoptosis (Reproduced with permission from Tsujimoto, 1998).

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#### 2.4.2.1 Anti-apoptotic Proteins

The anti-apoptotic proteins are represented by the members of Bcl-2 subfamily which share BH1, BH2, BH3 and BH4 domains. In Bcl-2 and Bcl-xL proteins, the BH1 and BH2 domains form a hydrophobic pocket on the surface of the protein. The BH3 domains of the pro-apoptotic proteins bind into the pocket formed. A BH4 domain is found only in the anti-apoptotic Bcl-2 subfamily and is thought to be crucial for survival. It has been implicated in providing protection through the activation of survival signalling mechanisms (Adams & Cory, 1998; Cory et al., 2003).

Localisation of the anti-apoptotic Bcl-2 subfamily proteins varies. Their hydrophobic carboxy-terminal domain aids in targeting them to the cytoplasmic face of three intracellular membranes: the outer mitochondrial membrane, the endoplasmic reticulum (ER) membrane and the nuclear envelope. These anti-apoptotic proteins function mainly at the mitochondria to prevent the activation Bax and Bak and eventually block the process of apoptosis. More specifically, Bcl-2 has been shown to be localised to both the mitochondria and to the ER (Hacki et al., 2000). Mitochondria-localised Bcl-2 is able to protect against apoptosis by sequestering BH3-only proteins while ER-localised Bcl-2 by regulating $Ca^{2+}$ fluxes through the ER membrane (Bassik et al., 2004).

In recent years, proteins in Bcl-2 subfamily have been identified as drug targets in the design of new anti-cancer therapies. The anti-cancer agents are able to occupy the hydrophobic pocket of the anti-apoptotic proteins and mimic the function of the BH-3 only subset of pro-apoptotic members. Indeed, several BH3 mimetic compounds have been reported to block the function of anti-apoptotic proteins. ABT-737 is a BH3-mimetic compound that binds and inhibits Bcl-2, Bcl-xL and Bcl-w to induce apoptosis preferentially in cancer cells and not in normal cells (Oltersdorf et al., 2005).

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Another study suggested that Bcl-2 can be converted from an anti-apoptotic protein to a pro-apoptotic protein following the binding of Nur77 (Kolluri et al., 2008). Nur77 is an orphan nuclear receptor which belongs to the steroid/thyroid/retinoid nuclear receptor superfamily and plays important roles in regulating cell proliferation, differentiation and apoptosis (Lin et al., 2004; Han et al., 2006). This protein can move from the nucleus to the mitochondria and then interacts with Bcl-2 protein within its N-terminal loop region between BH3 and BH4 domains, to result in a conformational change. The conformational change eventually modifies the function of Bcl-2 as a pro-apoptotic protein (Thompson & Winoto, 2008). Thus, the activation of Nur77 from the nucleus to the mitochondria in cells overexpressing Bcl-2 is one of the methods suggested for induction of apoptosis.

#### 2.4.2.2 Pro-apoptotic Proteins

The pro-apoptotic proteins are represented by two subsets which possess BH1-3 domains or a BH3 domain only. Bax and Bak proteins have three BH domains. Bax normally resides in the cytosol as a monomer while Bak resides on the mitochondrial membrane (Wolter et al., 1997; Dewson et al., 2009). In response to chemotherapeutic agents, Bax undergoes a conformation change and integrates into the outer mitochondrial membrane. Homo-oligomerisation between Bax and Bak forms pores through which cytochrome c is released, leading to caspase activation and ultimately apoptosis (Mikhailov et al., 2003). The concurrent deletion of both Bak and Bax dramatically diminishes apoptosis in many cells and accordingly either Bak or Bax are essential for apoptosis (Upreti et al., 2008; Gillissen et al., 2010).

The second subset of pro-apoptotic proteins is BH3-only proteins and they cannot function alone to cause the release of cytochrome c. Many mechanisms involved in the activation of these proteins such as, increased transcription, protein stabilisation and post-



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translational modification (Happo et al., 2012). More specifically, Bim and Bmf proteins have been shown to be bound to actin or dynein motor complexes within microtubules and following microtubule disruption they are activated and released (Pinon et al., 2008); PUMA and Noxa are induced in response to p53 (Shibue et al., 2006); Bid is activated by proteolysis to tBid upon association of the death receptors with ligand (Yi et al., 2003; Kantari & Walczak, 2011) Bad is activated by dephosphorylation in response to removal of growth factor (Tsang et al., 2008). The BH3-only proteins act upstream of Bax and Bak, and require either Bax or Bak to trigger apoptosis.

There are two distinct models proposed on how activation of Bax and Bak occur, direct and indirect activation, as shown in Figure 2.12. The direct activation model suggests that only certain BH3-only proteins such as, Bim and Bid can bind Bax and Bak directly and promote their activation (Chipuk et al., 2003; van Delft & Huang, 2006; Dewson & Kluck, 2009). Other BH3-only proteins such as, PUMA, Noxa and Bad are called as sensitisers as they cannot bind to and directly activate Bax and Bak (Cassidy-Stone et al., 2008), however, they bind to the anti-apoptotic proteins thus displacing the activators that enable them to activate Bax and Bak (Basañez & Hardwick, 2008; Tait & Green, 2010). In the indirect activation model, Bax and Bak must be bound by anti-apoptotic proteins to prevent their activation (Adams & Cory, 2007; Leslie, 2009). It is proposed that all of the BH3-only proteins can target and then neutralise the anti-apoptotic proteins, allowing for the activation of Bax and Bak. However, the mechanism by which Bax and Bak become activated as stated in this model is still controversial.

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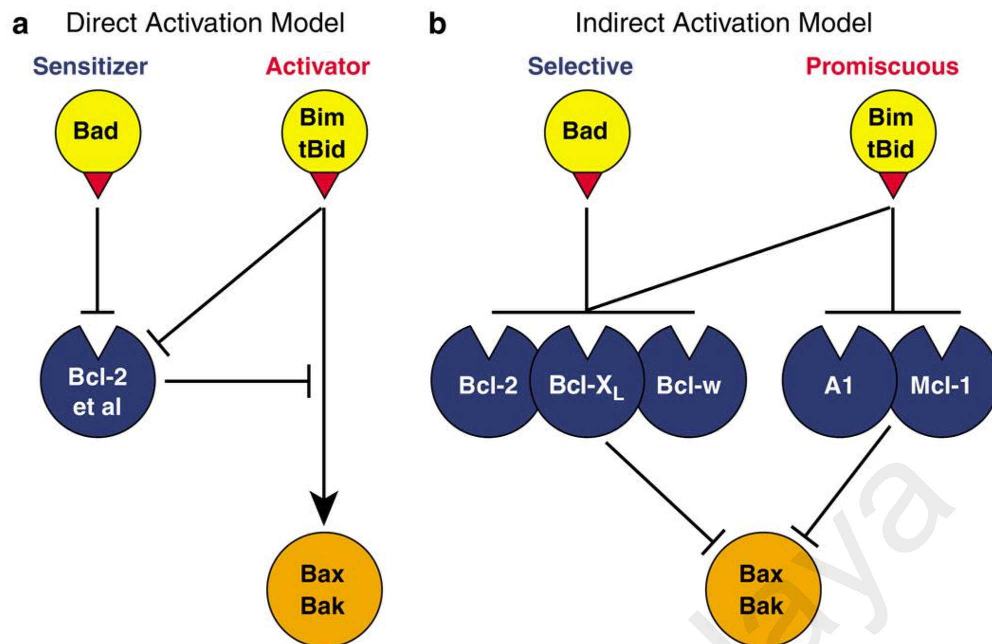


**Figure 2.12**: Comparison of direct and indirect activation models for Bax and Bak (Reproduced with permission from Adams & Cory, 2007).

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### 2.4.3 Role of p53 in Apoptosis

p53 is the most extensively studied tumour suppressor protein encoded by the Trp53 gene which plays an important role in response to diverse forms of cellular stress to mediate various anti-proliferative processes. One of the most important p53 functions is its ability to induce DNA damage apoptosis. It has been reported that p53 tumour suppressor proteins evoke apoptosis by upregulating the expression of pro-apoptotic Bax in response to sensing DNA damage. Activated Bax proteins in turn, stimulates mitochondria to release cytochrome c and catalyses downstream apoptotic events (Hanahan & Weinberg, 2000). Ser15 phosphorylation is required for p53 transcriptional activity and interaction with p300, the p53 transcriptional co-activator (Dumaz & Meek, 1999).

The first reference about p53 could control apoptosis came from the study by Oren and collaborators who reintroduced p53 into a p53-deficient myeloid leukaemia cell line (Yonish-Rouach et al., 1991). Here, the apoptotic induction effect of p53 could be counteracted by interleukin-6, the prosurvival cytokine. Later, another study using thymocytes from p53 knockout mice showed that p53 was required for radiation-induced apoptosis, but only when it is induced by stimuli that cause DNA damage (Clarke et al., 1993; Lowe et al., 1993b).

In addition to its role in inhibiting tumourigenesis, p53-dependent apoptosis is responsible for the chemotherapy-induced cell death (Johnstone et al., 2002). This was first performed in studies using oncogenically transformed cells treated *in vitro* and *in vivo* (Lowe et al., 1993a) and was later extended to various settings. Coherent with the potential role for p53 in modulating chemotherapy in human cancers, loss of p53 function can promote chemoresistance in certain tumour types (Wallace-Brodeur & Lowe, 1999; Johnstone et al., 2002).

Collectively, p53 serves as a regulator of the apoptotic process that can regulate several key control points in both extrinsic and intrinsic pathways as depicted in Figure 2.13. Thus, a better understanding of the p53 apoptotic program will provide insights for improved treatment for cancer.

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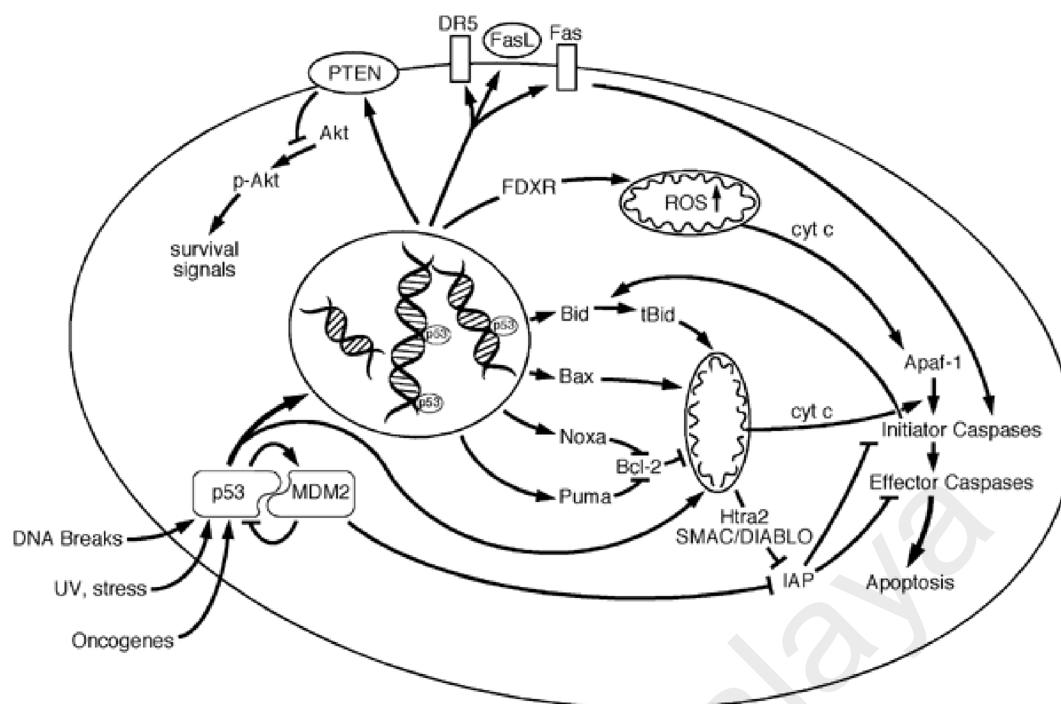


**Figure 2.13:** A model for p53-induced apoptosis by simultaneous targeting of several points in the apoptotic network (Reproduced with permission from Fridman & Lowe, 2003).

### 2.4.4 Role of PARP Cleavage in Apoptosis

Poly(ADP-ribose) polymerase (PARP) has been identified as a nuclear enzyme in the 1960's (Chambon et al., 1963; Sugimura et al., 1967). The PARP family consists of 17 distinct proteins found in eukaryotic cells; however, only PARP-1, PARP-2 and PARP-3 are known to function in the repair of single stranded DNA (Schreiber et al., 2006; Hassa & Hottiger, 2008). A main mechanism by which PARP-1 is activated via breaks in the DNA strand induced by metabolic, chemical or radiation. Following the detection of single stranded break, PARP-1 recruits and activates enzymes needed to repair the damaged strand via the transfer of ADP-ribose molecules from $NAD^+$ to itself and other key members of the DNA repair machinery (Haince et al., 2007; Banerjee & Kaye, 2011).



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Due to its activation by binding with DNA ends or strand breaks, PARP was postulated to contribute to cell death by the depletion of NAD and ATP in the cells (Berger & Petzold, 1985). PARP was the first cellular protein to be discovered as being specifically cleaved in apoptosis (Kaufmann, 1989), and its cleavage subsequently was found to be a common phenomenon occurring in apoptosis induced by different types of stimuli (Kaufmann et al., 1993). Specific proteolysis of PARP occurs resulting in the formation of 89- and 24-kDa fragments that contain the active site and the DNA-binding domain of the enzyme, respectively, during drug-induced apoptosis in various cell types (Kaufmann et al., 1993; Nicholson et al., 1995). Such cleavage essentially abolishes the PARP's catalytic activity in response to DNA damage (Tewari et al., 1995). Caspases are responsible for the PARP cleavage after the apoptotic cascade has been triggered (Nicholson & Thornberry, 1997). Although caspase-3 and caspase-7 are the most powerful proteases for PARP cleavage, all caspases found to date are able to cleave PARP with certain degrees of efficiency (Ghayur et al., 1997).

## 2.5 Metastasis

Cancer metastasis is the process where cancer cells spread from primary tumour to other tissues and organs, and finally the formation of new tumours (Chaffer & Weinberg, 2011). At the time of cancer diagnosis, about 50% of the patients already present clinically detectable metastatic disease (DeVita et al., 1975). Metastatic disease remains the most common cause for cancer-associated deaths (Siegel et al., 2017). In order for the tumour cells to metastasise successfully, they must undergo complex cascade of events called metastatic cascade. This cascade can be generally separated into three main processes: invasion, intravasation and extravasation (Figure 2.14). The loss of cell-cell adhesion ability allows malignant tumour cells to detach from the primary tumour mass and changes in cell-matrix interaction facilitate the cells to undergo the process of

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invasion through the basement membrane. The process of intravasation involves the dissociated cells to enter the blood and lymphatic vessels in their vicinity and then metastasise to distant sites. Once the tumour cells have arrived at a likely point of intravasation, they interact with the endothelial cells by undergoing biochemical interactions to form stronger bonds, and through the process of extravasation invade the vascular endothelium and the basement membrane. These cells can then proliferate and stimulate blood vessel growth stimulation through a process known as angiogenesis to allow micrometastases growth into macroscopic tumours at this secondary focus (Ma & Weinberg, 2008; Bracken et al., 2009).

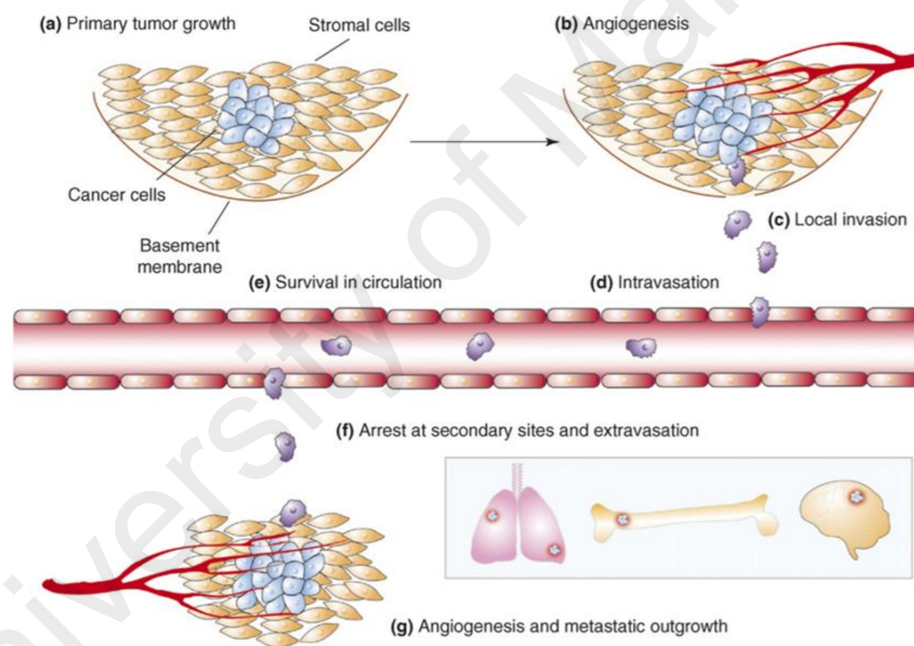


**Figure 2.14:** Steps in the metastatic process (Reproduced with permission from Ma & Weinberg, 2008).

### 2.5.1 Cancer Invasion and Epithelial-Mesenchymal Transition

The dynamic reorganisation of actin cytoskeleton is a precondition for migration and invasion of cancer cells, with the formation of membrane protrusions leading to the mesenchymal phenotype (Yilmaz & Christofori, 2009).

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Cadherins are transmembrane proteins that mediate cell adhesion through extracellular domain, while the intracellular domain regulates signalling to the actin cytoskeleton (Halbleib & Nelson, 2006). Epithelial E-cadherin is a protein expressed on epithelial cells with the function of mediating cell-cell and cell-matrix adhesion. The expression of neural N-cadherin on cancer cells plays a role in formation of contact between cancer cell-endothelial wall and consequently activates Src-kinases activity to promote transendothelial migration (Ramis-Conde et al., 2008).

Cell invasion involves the remodelling of extracellular matrix (ECM) and rearrangement of basement membrane. EMT program has been suggested as the crucial mechanism for the acquisition of malignant phenotypes by epithelial cancer cells (Yilmaz & Christofori, 2009). EMT is typically characterised by the downregulation of E-cadherin and the upregulation of N-cadherin, which also known as "cadherin switch" (Araki et al., 2011). EMT is orchestrated by many transcription factors such as, Snail1, zinc finger E-box binding homeobox (ZEB) and TWIST families that regulate actin cytoskeletal remodelling and ECM protein degradation by ECM-degrading proteases for examples matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA). Actin cytoskeleton remodelling and ECM degradation allow invasion of cells into the stroma and intravasation into the blood or lymphatic circulation. The cells then travel to the regional lymph nodes or distant organs. Later, a reversal event known as mesenchymal-epithelial transition (MET) with increased expression of epithelial-specific gene and suppressed expression of mesenchymal-specific gene, thus leading to the formation of macroscopic metastases with epithelial characteristic at secondary site (Bracken et al., 2009; Baranwal & Alahari, 2010). The role of EMT in cancer metastasis is illustrated in Figure 2.15.

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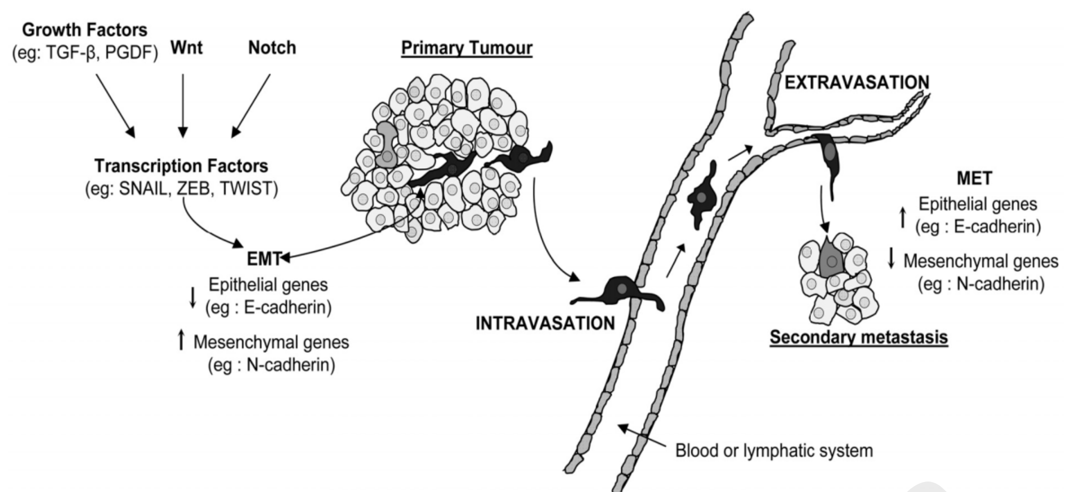


**Figure 2.15:** Overview of the role of EMT in tumour metastasis (Reproduced with permission from Bracken et al., 2009).

### 2.5.2 Cancer Migration

Cell migration is an important event during tumour invasion and metastasis progression (Stetler-Stevenson et al., 1993). The major structures of the actin cytoskeleton involved in different steps of cell migration are lamellipodia, filopodia, focal adhesion and lamella (Le Clainche & Carlier, 2008). The tumour cells use migration mechanism that are similar to those occur in the process of normal cells such as, embryonic morphogenesis, inflammatory immune responses, wound healing and angiogenesis (Friedl & Brocker, 2000).

To migrate, a cell first extends protrusions in the direction of migration in respond to migration-promoting agent. The large and broad lamellipodia or spike-like filopodia protrusions at the leading edge are driven by actin polymerisation and stabilised by adhesion with extracellular matrix or adjacent cells via transmembrane receptors that are linked to the actin cytoskeleton. Next, forward extension of lamellipodium by adhering to the surface of leading edge, while de-adhesion at the cell body and retraction of the trailing edge resulting in a net translocation of the cell in the direction of the movement.



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Furthermore, actin filaments must be disassembled at the trailing end, so that actin monomers can be replenished for further polymerisation at the leading edge (Pollard & Borisy, 2003; Ridley et al., 2003; Ananthakrishnan & Ehrlicher, 2007). The three stages of cell migration are shown in Figure 2.16.

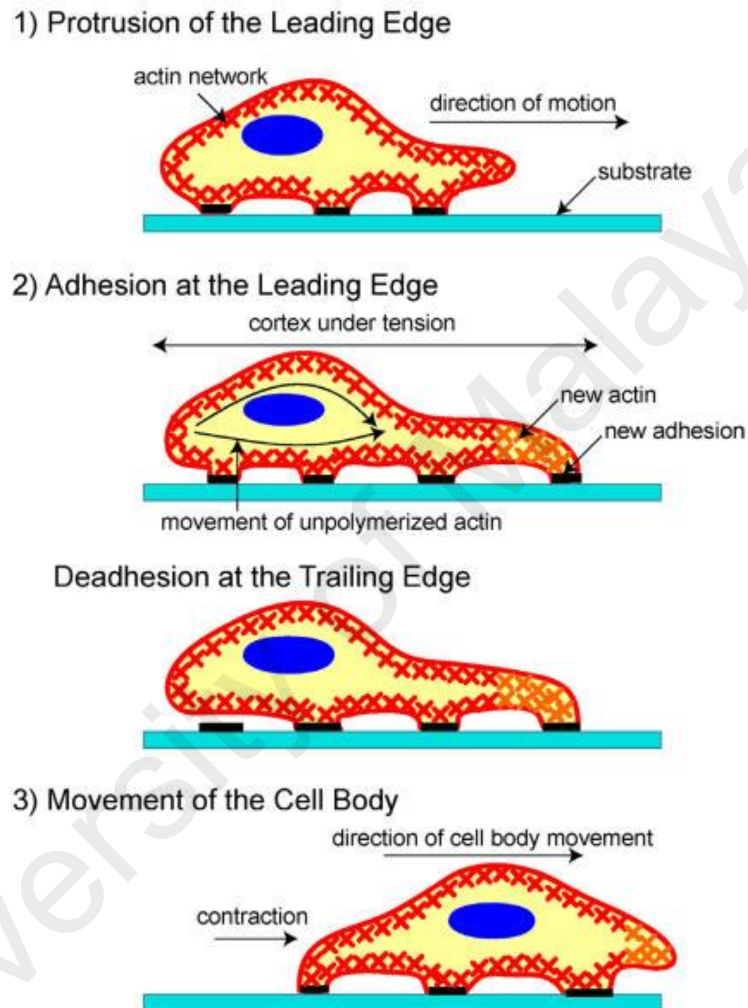


**Figure 2.16:** A schematic of cell migration (Reproduced with permission from Ananthakrishnan & Ehrlicher, 2007).

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### 2.5.3 Integrin-FAK-Src Signalling Transduction

The regulation of cell migration involves a complex interaction between extracellular matrix, transmembrane receptors, kinases, adapter proteins and other downstream

signalling molecules that triggers cell morphological changes (Friedl & Brocker, 2000). Integrin-focal adhesion kinase (FAK)-Src signalling transduction participates in the regulation of metastasis by loosening cell-ECM adhesion and thus promoting cell invasion and migration (Hood & Cheresh, 2002). Integrins are transmembrane heterodimeric proteins consisting of α and β subunits that function in tethering cells to the ECM (Hynes, 2002). The activation of integrin induces recruitment and activation of FAK and Src protein tyrosine kinase to trigger integrin-FAK-Src intracellular transduction cascades. The stimulated FAK and Src target multiple downstream proteins including the Rho GTPases superfamily proteins such as, RhoA, Rac1 and Cdc42 GTPases, thereby contributing to the migratory and invasive phenotype via reorganisation of the actin cytoskeleton (Hood & Cheresh, 2002; Schneider et al., 2008). During cell movement, Cdc42 mediates assembly of long, thin, actin-containing extensions called filopodia; Rac mediates formation of curtain-like extensions called lamellipodia and ruffles; whereas RhoA activation regulates formation of stress fibres and focal adhesion of cells to induce retraction of the trailing edge (Nobes & Hall, 1995). The overview of integrin-FAK-Src signalling pathway is illustrated in Figure 2.17.

The growing evidence has highlighted the implication of integrin signalling transduction cascade in cancers and metastasis progression. Several experimental models have shown that targeting integrin β1 could partly reduce the metastatic potential of cancer cell *in vitro* and *in vivo* (Wang et al., 1998; Park et al., 2006; Park et al., 2008). Hence, this suggested that integrin play a role in invasive and metastatic cancer properties. Furthermore, overexpression of Fak, the downstream signalling molecule of integrin signalling is associated with cancer metastasis where high Fak expression was found in metastatic cancer (Cance et al., 2000). Moreover, integrin β1 and FAK signalling directly regulate the proliferation and invasion of metastatic cells in the lung (Shibue & Weinberg, 2009).



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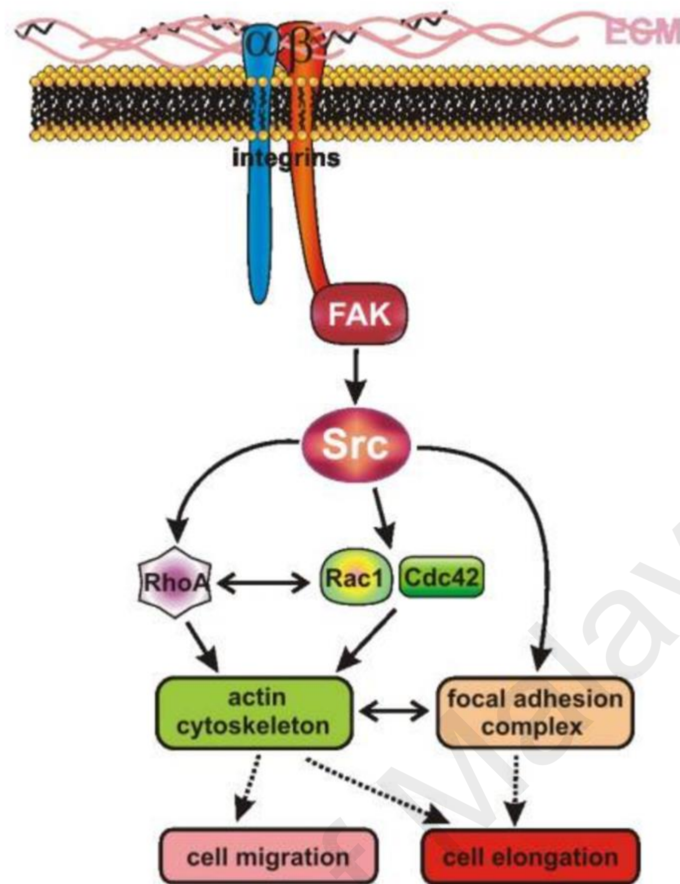


**Figure 2.17:** Schematic overview of the integrin-FAK-Src signalling transduction (Reproduced with permission from Schneider et al., 2008).

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### 2.5.4 PI3K/Akt Signalling Pathway

The integrin-dependent adhesion signalling has an impact on the activation of phosphatidylinositol 3-kinase (PI3K)-AKT pathway (Sieg et al., 1999). The PI3K/Akt signalling pathway is often dysregulated in cancer (Thorpe et al., 2015). Indeed, this signalling pathway plays a vital role in regulating cell proliferation, survival or migration of cancer cells. To date, anti-cancer agents that target PI3K or Akt have been widely developed and are being tested in clinical trials (Wong et al., 2010; Rodon et al., 2013).

PI3Ks, constitute a lipid kinase family mostly generate phosphatidylinositol (3,4,5) trisphosphates (PIP3) in response to ligand stimulation of growth factor receptor kinases

(GFRKs), integrin, G protein-coupled receptors (GPCR) and cytokine receptor (CR). The oncogenic serine/threonine kinase Akt, is recruited to the cell membrane by interaction with PIP3 via its pleckstrin homology (PH) domain, being phosphorylated at two residues (Thr308 and Ser473) by phosphoinositide-dependent kinase 1 (PDK1), PDK2 and mTOR complex 2 (mTOR2) (Xue & Hemmings, 2013). Activated Akt controls diverse cellular functions including cell survival, growth, cell migration and angiogenesis (Manning & Cantley, 2007). The PI3K/Akt signalling pathway is illustrated in Figure 2.18.

Accumulating evidence is emerging that PI3K/Akt signalling pathway actively involves in the migratory process of metastatic cancer cells via phosphorylation of several cytoskeleton-regulating proteins and EMT-activating proteins. For instance, it has been shown that cells with a constitutively active form of Akt produce a transcription factor, Snail, which is known to repress expression of the E-cadherin gene (Grille et al., 2003). This transcriptional activity induces cellular changes leading to the conversion of epithelial cells into invasive mesenchymal cells. In addition, Akt also induces the production of metalloproteinases and leading to cell invasion (Kim et al., 2001; Park et al., 2001).

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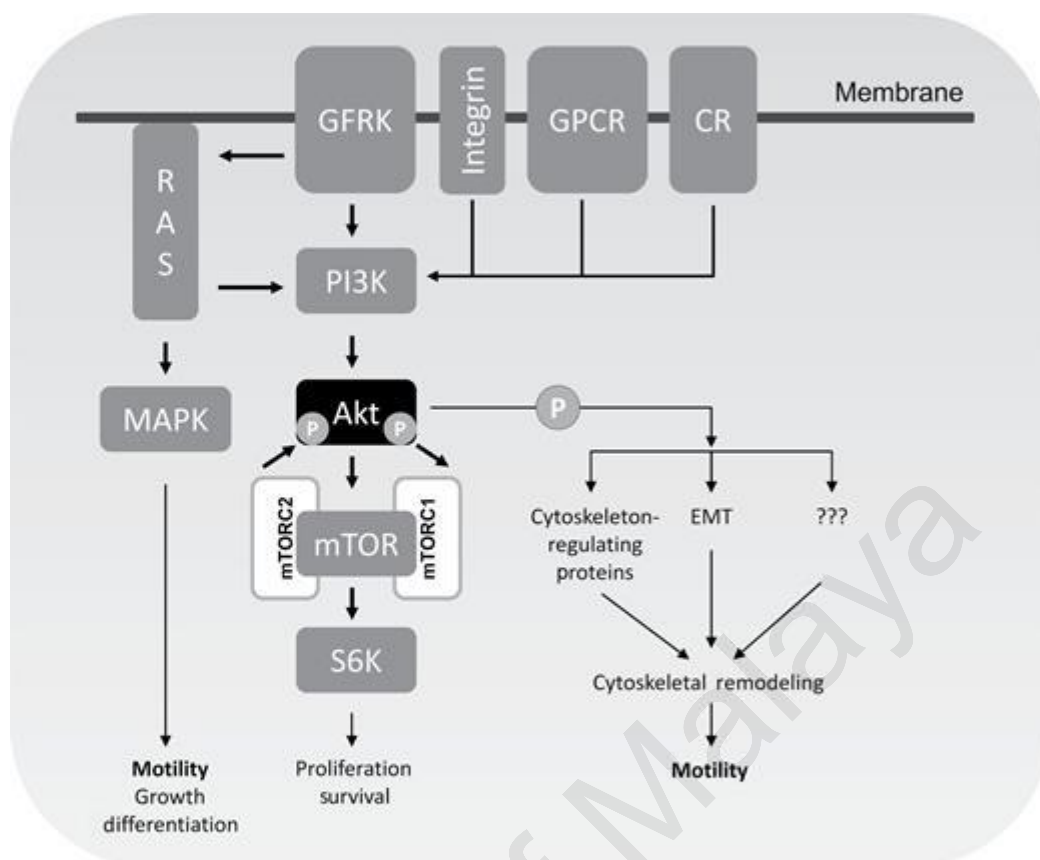


**Figure 2.18:** Model for the regulation of PI3K/Akt signalling pathway (Reproduced with permission from Xue & Hemmings, 2013).

## 2.6 Structure-Activity Relationship (SAR)

A structure-activity relationship (SAR) relates features of a chemical structure to a biological activity associated with that chemical. Biological properties of new compounds are often inferred from profiles of similar existing compounds whose biological mechanism are already known. In the pharmaceutical and chemical industries, SARs have been widely used to design chemicals with commercially desirable properties. SAR develops an understanding of what constitutes a class of molecules that are active, what determines relative activity, and what distinguishes these from inactive classes (McKinney et al., 2000). In the field of cancer research, SAR is analysed in order to find new, more effective anti-cancer agents. A number of literature reviews have addressed

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the SAR of anti-cancer agents in terms of inhibition of cancer cell growth, however SAR on targeting the metastatic process has not received as much attention. Therefore, it is important to study the SAR of different functional groups in natural product and synthetic analogues with not only their anti-proliferative but anti-metastatic properties.

### 2.6.1 SAR on Anti-Proliferation Effects

In particular, a series of compound containing different substituents was synthesised as a continuation or ongoing anti-cancer research development. The target compounds were evaluated for anti-proliferative activities against several types of cancer. SAR study has been made to correlate between the chemical structures and anti-proliferation activities. In this study, the functional groups including acetoxy, methoxy and hydroxy groups are being emphasised. Various studies showed that they may exert either effective or weak anti-proliferative effects on different cell lines. Moreover, the position of the functional groups may also affect the effectiveness of the substituent in blocking the growth of cancer cells.

#### 2.6.1.1 Acetoxy ($CH_3COO$) Group

Acetoxy group is a chemical functional group of the structure $CH_3$-C(=O)-O-. Based on the inductive effect, the acetoxy group is electron withdrawing.

A series of aspirin-based benzyl esters (ABEs) were synthesised and their inhibitory activity against human colon (HT-29 and SW480) and pancreatic (BxPC-3 and MIA PaCa-2) cancer cell lines was evaluated (Joseph et al., 2011). The acetoxy substituent showed appreciable potency in inhibiting cancer cell growth. Surprisingly, the $IC_{50}$ of the *para* substituted acetoxy compound was 5 to 20-fold lower than that of the *p*-aspirin containing compound.

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Evaluation for the anti-cancer activity of C7-oxgenated spongiane diterpenes derivatives was performed on HeLa and HEp-2 (HeLa derivative) cell lines. In general, introduction of acetoxy group at C-7 did not improve the resultant cytotoxicity (Arno et al., 2003). Hence, acetoxy group may not be effective in the anti-proliferative effects.

Another SAR study that has examined the effect of coumarin analogues on three human cancer cell lines have shown that substitution of the hydroxy group with an acetoxy group reduced the cytotoxic activity. However, some analogues displayed a minimal cytotoxic activity with the introduction of acetoxy group (Miri et al., 2016).

In short, introduction of acetoxy group remains important in the development of anti-proliferative properties of compounds.

#### 2.6.1.2 Methoxy ($OCH_3$) Group

A methoxy group consists of a methyl group bound to oxygen, with the formula O–$CH_3$. The Hammett equation classifies a methoxy substituent on a benzene ring as an electron-donating group (Hammett, 1937).

Analysis of SAR of a series of (Z)-1-(1,3-diphenyl-1H-pyrazol-4-yl)-3-(phenylamino)prop-2-en-1-one derivatives revealed that methoxy group as electron donating group attached to the aromatic B ring could contribute to significant cytotoxicity (Srinivasa et al., 2016). Analogues having methoxy substitution at B ring and analogues with 3,4,5,-methoxy substitution, all showed better $IC_{50}$ values than other analogues with electron withdrawing group.

Sreelatha and collaborators studied a series of novel naphthoquinone amide derivatives for the anti-cancer activity against HeLa and SAS cancer cell lines (Sreelatha et al., 2014). Among the analogues synthesised, compounds with the presence of methoxy

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substituent at C-2 of the quinone ring showed active activity. Moreover, the attachment of methoxy group at C-5 position reduced the anti-proliferation activity. Not only is the methoxy group essential for anti-cancer activity but the position of this group plays an important role in the effectiveness of the compound to inhibit growth of cancer cells.

To obtain data on the SAR in the imidazobenzothiazole series, Trapani and friends examined several analogues for their cytotoxic effects (Trapani et al., 2001). Introduction of methoxy group at C-7 position could give better activity if compared to their parent compound. However, additional substitution of methoxy group at 5- and 8- position caused the reduction of cytotoxic activity compared to the mono-methoxy analogue. Hence, the more methoxy group substituted to the compound, the less efficient is the ability to inhibit the growth of cancer cells.

Overall, methoxy group is considered as an important functional group in exerting anti-proliferation effects, however its positions and the number of substituent influence its effectiveness.

#### 2.6.1.3 Hydroxy (OH) Group

A hydroxy or hydroxyl group is a substituent with the formula OH. It is classified as the electron donating group.

In 2016, a series of (Z)-1-(1,3-diphenyl-1H-pyrazol-4-yl)-3-(phenylamino)prop-2-en-1-one derivatives were synthesised and analysed for their anti-cancer effects against HT-29, PC-3, A549 and U87MG human cancer cell lines (Srinivasa et al., 2016). SAR analysis showed that hydroxy group substituted on the aromatic B ring enhanced the activity of analogues.

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Evaluation of the structure-activity relationships of a series of novel fluorinated asiatic acid analogues were carried out based on their anti-proliferation activity against HeLa and HT-29 cell lines (Goncalves et al., 2016). The compound with three free hydroxy groups in A-ring exhibited lower anti-proliferation activity when compared with compound which had two free hydroxy groups. However, when the two hydroxy groups in compound are acetylated, the activity increased. These combined results indicated that free hydroxy groups in A-ring are not important for the anti-proliferation activity.

Hence, substitution of hydroxy group to the compound may exert anti-proliferation effects to certain extent.

### 2.6.2 SAR on Anti-Migration Effects

Various approaches have been performed to study the anti-migration effects of a series of synthesised compound. The details of SAR on anti-migration activities provide better insights in the design and development of improved anti-cancer drug with anti-metastatic property. The anti-migration effects of electron withdrawing group which represented by acetoxy group and electron donating group including methoxy and hydroxy group were discussed in this study.

#### 2.6.2.1 Acetoxy ($CH_3COO$) Group

Mishkinee and friends investigated the anti-metastatic activity of derivatives of alkylthiocarboxylic acids. They showed that the absence of the acetoxy group leads to a significant drop in this activity (Mishkinene & Valavichene, 1997). In other words, attachment of acetoxy substituents improves the anti-migration activity.

A study of betulin derivatives on the anti-invasive activity on prostate cancer cells reported that when acetoxy group substituted at position C28, anti-invasive activity was

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decreased (Härmä et al., 2015). Succinctly, acetoxy group is unfavourable for anti-invasive effects.

Due to the limited references, it is difficult to conclude the effectiveness of acetoxy group in modulating the anti-migration activity.

#### 2.6.2.2 Methoxy ($OCH_3$) Group

In the study by Tseng and friends, the compound (E)-6-methoxy-3-(4-methoxyphenyl)-2-[2-(5-nitrofuran-2-yl)vinyl]quinoline was found to be weakly cytotoxic in all the cancer and normal cells investigated, but had the ability to inhibit the migration and invasion of cells (Tseng et al., 2015). It can therefore be deduced that the methoxy group substituted at C-6 position of the quinoline ring is crucial for anti-metastatic activity. Methoxy groups have also been found in the structure of combretastatin A4 (CA-4), a known anti-angiogenesis agent (Griggs et al., 2002). Thus, methoxy groups are potential contributors to the anti-metastatic effects.

In another study, a series of EF24 analogues were synthesised and analysed for their anti-cancer activity against three different cell lines of lung cancer, A549, LLC and H1650 (Wu et al., 2017). Most of the compounds showed good anti-proliferation activity. Among them, a compound with three methoxy groups attach to its side showed greater cytotoxicity than original analogue, EF24. It also exhibited good anti-migration effect against A549 cells. Hence, the methoxy group plays a major role in the anti-metastatic activity of cancer treatment.

Cathepsins, the cysteine proteases involved in the progression of various human cancers are promising therapeutic target in cancer treatment (Gocheva & Joyce, 2007). This is because the inhibition of one of its members, cathepsin L reduced cancer cell invasion and migration (Lankelma et al., 2010). In 2013, Parker and friends carried out



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the synthesis of benzoylbenzophenone thiosemicarbazone derivatives and assessed for the inhibitory activity against cathepsins L (Parker et al., 2015). The activity was diminished when the compound was substituted with the methoxy group. They suggested that the substitution increased the steric hindrance and thus reduced the activity. The *para* substituted methoxy analogue exhibited high $IC_{50}$ value of 5117 nM and significantly weaker than the unsubstituted analogue. Thus, it was concluded that methoxy group does not have an important role in this activity.

Taken together, methoxy substituent may help to inhibit the migration of cancer cells, unless its effect is being influenced by steric hindrance.

#### 2.6.2.3 Hydroxy (OH) Group

SAR study on brartemicin analogues conducted by Jiang and friends showed that anti-invasive activity to be moderately active when hydroxy group was substituted at the 2- or 4-position of the benzoic acid ring (Jiang et al., 2011). The hydroxy substituted analogues maintained the anti-invasive activity at an $IC_{50}$ of not more than 1.0μg/ml, although they were slightly less potency compared to the natural compound.

Benzoylbenzophenone thiosemicarbazone analogues were evaluated for the inhibitory activity against cathepsins L (Parker et al., 2015). It was observed that *para* substitution of hydroxy at the analogue resulted in diminished activity with the $IC_{50}$ value of 340 nM which less potent than original analogue. The reduced activity was thought to be due to the steric hindrance by *para* hydroxy group. Hence, it is important to select suitable substituent in order to reduce the steric hindrance effects to the anti-cancer activity.

Andrographolide derivatives were evaluated to identify potent inhibitors against cancer cell migration and invasion (Wu et al., 2013). Analogue in which the allylic hydroxy at C-14 position was removed had better inhibitory effects on migration in

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human bladder carcinoma 5637 cells than original compound. In other words, the hydroxy group may hinder the anti-migration effect on cancer cells. This has to be further validated.

In summary, hydroxy group is not expected to display strong anti-migration on cancer cells.

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# CHAPTER 3: MATERIALS AND METHODS

## 3.1 ACA and Its Analogues

### 3.1.1 General Chemistry Procedure

Unless otherwise noted, all materials were obtained from commercial suppliers and were used without further purification. Reaction time and purity of products were monitored by thin layer chromatography (TLC) on Merck silica gel aluminium cards (0.2 mm thickness) with fluorescent indicator at 254 nm. Column chromatography was run on silica gel (200–300 mesh) obtained from EMD Millipore (USA). Structural elucidation was established through following spectroscopic methods: IR on a Perkin Elmer RX1 FT-IR spectrometer, UV on a Shimadzu UV-160A UV–Visible Recording Spectrophotometer, MS on a Shimadzu gas chromatograph-MS spectrometer (HP 6890 Series Mass Selective Detector and HP 6890 Series GC System), 1D ($^{1}H$, $^{13}C$, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra using $CDCl_3$ as solvent were recorded on Bruker AVN 400 (400 MHz for $^{1}H$ NMR, 100 MHz for $^{13}C$ NMR) spectrometer.

### 3.1.2 Plant Materials

The rhizomes of *Alpinia conchigera* Griff. were collected from Jeli province of Kelantan, east-coast of Peninsular Malaysia. The sample was identified by Professor Dr. Halijah Ibrahim from the Division of Ecology and Biodiversity, Institute of Biological Science, Faculty of Science, University of Malaya. A voucher specimen (KL5049) was deposited in the Herbarium of Chemistry Department, Faculty of Science, University Malaya.

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### 3.1.3 Isolation of 1'S-1'-acetoxychavicol acetate (ACA)

Isolation of the ACA was carried out by Mr. Mohamad Nurul Azmi from the Department of Chemistry, Faculty of Science, University Malaya. The air-dried and grounded rhizomes of *Alpinia conchigera* (2.10 kg) were extracted successively with hexane ($C_6H_{14}$), dichloromethane ($CH_2CL_2$) and methanol (MeOH) at room temperature (25°C) for 72 hrs. The solvent suspension was filtered and concentrated to dryness by using a rotary evaporator in order to obtain crude extract. Isolation and purification of ACA within the crude extract was examined using column chromatography (CC) and TLC techniques. The crude was subjected to CC ($SiO_2$, 230-400 mesh; n-hexane/ethyl acetate step gradient [100 → 50; % n-hexane]) to obtained 20 sub-fractions base on TLC profile (Aluminium supported silica gel 60 F254 plates; n-hexane/ethyl acetate [80:20]): AM1.Fr1-AM1.Fr20. From this crude, fraction AM1.Fr6 was confirmed as a target compound *i.e.* 1'S-1'-acetoxychavicol acetate (ACA). The structure of the isolated ACA was determined by comparison of the spectral data of mass and nuclear magnetic resonance with those reported in the literatures (Janssen & Scheffer, 1985; Yang & Eilerman, 1999; Ando et al., 2005). The spectroscopic summary of ACA: A yellowish oil, Calculated for $C_{13}H_{14}O_4$, 234.2479; Found 234, 192, 150, 149, 132, 104, 77. IR (neat) $\upsilon_{max}$, $cm^{-1}$: 1761, 1645, 1234. UV $\lambda_{max}$, nm: 304.5. $^1H$ NMR ($CDCl_3$, 400 MHz), δ: 2.08 (3H, *s*), 2.27 (3H, *s*), 5.22 (2H, *dd*, $J$ = 9.98 Hz), 5.98 (1H, *m*), 6.23(1H, *d*, $J$ = 5.84 Hz), 7.03 (2H, *d*, $J$ = 8.80 Hz), 7.33 (2H, *d*, $J$ = 8.76 Hz). $^{13}C$ NMR ($CDCl_3$, 100 MHz), δ: 21.2 ($CH_3$), 21.3 ($CH_3$), 75.6 (CH), 117.2 ($CH_2$), 121.7 (2CH), 128.5 (2CH), 136.1 (CH), 136.5 (C), 150.5 (C), 169.4 (C), 169.7 (C).

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### 3.1.4 General Procedure to Obtain Compounds 2-8

4-allyl-2,6-dimethoxyphenol (**2**), (*S*)-α-vinylbenzyl alcohol (**3**) and 4-allyl anisole (**5**) were purchased from Sigma-Aldrich (USA). Eugenol (**4**) was purchased from Merck (USA). Compounds **6**, **7** and **8** were hemi-synthesised from **2**, **3** and **4** respectively by reacting with acetic anhydride in the presence of 4-dimethylaminopyridine (catalyst) and dichloromethane (solvent) (Figure 3.1).

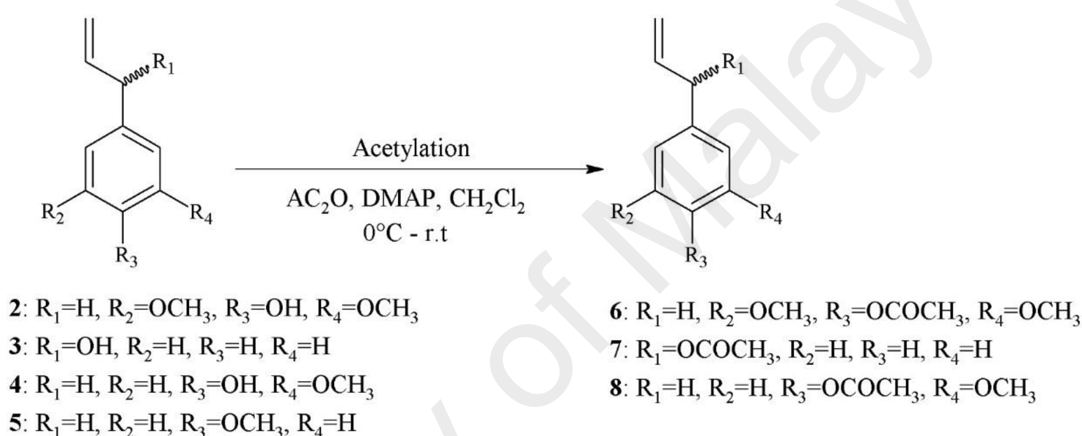


**Figure 3.1:** Schematic preparation of compounds **2-8**.

### 3.1.5 General Procedure to Obtain Compounds 17-20

Compounds **17-20** were hemi-synthesised by modification of Lee's procedure (Lee & Ando, 2001), as shown in Figure 3.2.

#### 3.1.5.1 1'-acetoxyeugenol acetate (17, AEA)

To a solution of vinylmagnesium bromide freshly prepared from magnesium turning (0.24 g, 9.87 mmol), 1M vinyl bromide (6.60 ml, 6.57 mmol) and catalytic amount of iodine crystal in dry diethyl ether (20.0 ml), a solution of aldehyde **9** (0.50 g, 3.29 mmol) in diethyl ether (10.0 ml) under ice bath was added. The ice bath was removed and the

mixture was allowed to warm to room temperature. After the reaction mixture was stored for 2 hrs at room temperature, saturated aqueous $NH_4Cl$ (10.0 ml) solution was added. The mixture was extracted with diethyl ether (2 x 20.0 ml). The resulting organic extracts were combined, and the solvent was removed under reduced pressure to yield a crude product. The oily product (intermediate **13**) obtained by usual work-up was acetylated with acetic anhydride (0.62 ml, 6.57 mmol) and 4-dimethylaminopyridine (DMAP) (0.81 g, 6.64 mmol) in dichloromethane (15.0 ml) in an ice bath. After consumption of starting material and product formation, the reaction was quenched with saturated aqueous $NH_4Cl$ (10.0 ml) solution and extracted with dichloromethane (3 x 15.0 ml), dried ($NaSO_4$) and filtered. Evaporation of filtrate gave a yellow oil which was purified by column chromatography on silica gel, and eluting with hexane/ethyl acetate (9:1) afforded the desired product as a yellowish oil **17** (0.53 g, 61%). The synthetic analogue **17** is the same as the natural analogue AEA that was previously studied (Hasima et al., 2010; In et al., 2011). The spectroscopic summary of analogue **17**: A yellowish oil, Calculated for $C_{14}H_{16}O_5$, 264.0993; Found 40, 45, 61, 77, 91, 107, 121, 264. IR (neat) $\upsilon_{max}$, $cm^{-1}$: 1767, 1742, 1647, 1607, 1233, 1198, 857. UV $\lambda_{max}$, nm: 299.0. $^{1}H$ NMR ($CDCl_3$, 400 MHz), δ: 2.12 (3H, *s*), 2.31 (3H, *s*), 3.84 (3H, s), 5.25 (2H, *dd, J*=10.5), 5.99 (1H, *m*), 6.24 (1H, *d*, *J*=5.80 Hz), 6.95 (1H, *s*), 7.02 (2H, *d*, *J*=7.80 Hz). $^{13}C$ NMR ($CDCl_3$, 100 MHz), δ: 20.6 ($CH_3$), 21.25 ($CH_3$), 55.9 ($OCH_3$), 75.7 (CH), 111.5 (CH), 117.0 ($CH_2$), 119.6 (CH), 122.8 (CH), 135.9 (CH), 137.6 (C), 139.7 (C), 151.2 (C), 168.9 (C), 169.7 (C).

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#### 3.1.5.2 1'-acetoxy-3,5-dimethoxychavicol acetate (18, AMCA)

To a solution of vinylmagnesium bromide freshly prepared from magnesium turning (0.17 g, 7.14 mmol), 1M vinyl bromide (3.6 ml, 3.57 mmol) and catalytic amount of iodine crystal in dry diethyl ether (20.0 ml), a solution of aldehyde **10** (0.30 g, 1.65 mmol) in diethyl ether (10.0 ml) under ice bath was added. The ice bath was removed and the

mixture was allowed to warm to room temperature. After the reaction mixture was stored for 1 hr at room temperature, saturated aqueous $NH_4Cl$ (10.0 ml) solution was added. The mixture was extracted with diethyl ether (2 x 20.0 ml). The resulting organic extracts were combined and solvent was removed under reduced pressure to yield the crude product. The oily product (intermediate **14**) obtained by usual work-up was acetylated with acetic anhydride (0.33 ml, 3.30 mmol) and DMAP (0.41 g, 3.36 mmol) in dichloromethane (15.0 ml) in an ice bath. After consumption of starting material and product formation, the reaction was quenched with saturated aqueous $NH_4Cl$ (10.0 ml) solution and extracted with dichloromethane (3 x 15.0 ml), dried ($NaSO_4$) and filtered. Evaporation of filtrate gave a yellow oil which was purified by column chromatography on silica gel, and eluting with hexane/ethyl acetate (9:1) afforded the desired product as a yellowish oil **18** (0.30 g, 63%). The spectroscopic summary of analogue **18**: A yellowish oil, Calculated for $C_{15}H_{18}O_6$, 294.1103. IR (neat) $\upsilon_{max}$, $cm^{-1}$: 1761, 1645, 1234. UV $\lambda_{max}$, nm: 304.5. $^1H$ NMR ($CDCl_3$, 400 MHz), δ: 2.13 (3H, *s*), 2.33 (3H, *s*), 3.81 (6H, *s*), 5.31 (2H, *dd*, *J* = 10.0 Hz), 5.98 (1H, *m*), 6.21 (1H, *d*, *J* = 5.80 Hz), 6.61 (2H, *s*). $^{13}C$ NMR ($CDCl_3$, 100 MHz), δ: 20.4 ($CH_3$), 21.2 ($CH_3$), 56.1 ($2OCH_3$), 76.0 (CH), 104.0 (2CH), 117.0 ($CH_2$), 128.4 (C), 135.9 (CH), 137.2 (C), 152.1 (2C), 168.7 (C), 169.9 (C).

#### 3.1.5.3 1'-acetoxy-3,5-dimethoxychavicol (19)

To a solution of vinylmagnesium bromide freshly prepared from magnesium turning (0.92 g, 38.3 mmol), 1M vinyl bromide (17.0 ml, 17.1 mmol) and catalytic amount of iodine crystal in dry diethyl ether (30.0 ml), solution of aldehyde **11** (0.98 g, 5.89 mmol) in diethyl ether (10.0 ml) under ice bath was added. The ice bath was removed and the mixture was allowed to warm to room temperature. After the reaction mixture was stored for 2 hrs at room temperature, saturated aqueous $NH_4Cl$ (10.0 ml) solution was added. The mixture was extracted with diethyl ether (2 x 30.0 ml). The resulting organic extracts

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were combined and solvent was removed under reduced pressure to yield the crude product. The oily product (intermediate **15**) obtained by usual work-up was acetylated with acetic anhydride (0.60 ml, 6.35 mmol) and DMAP (1.52 g, 12.4 mmol) in dichloromethane (20.0 ml) in an ice bath. After consumption of starting material and product formation, the reaction was quenched with saturated aqueous $NH_4Cl$ (10.0 ml) solution and extracted with dichloromethane (3 x 15.0 ml), dried ($NaSO_4$) and filtered. Evaporation of filtrate gave a yellow oil which was purified by column chromatography on silica gel, and eluting with hexane/ethyl acetate (9:1) afforded the desired product as a yellowish oil **19** (0.90 g, 65%). The spectroscopic summary of analogue **19**: A yellowish oil, Calculated for $C_{13}H_{16}O_4$, 236.1049. IR (neat) $\upsilon_{max}$, $cm^{-1}$: 1739, 1602, 1558, 1519, 1459, 1232. UV $\lambda_{max}$, nm: 304.5. $^1$H NMR ($CDCl_3$, 400 MHz), δ: 2.11 (3H, *s*), 3.77 (6H, *s*), 5.31 (2H, *dd*, *J* = 10.5 Hz), 5.97 (1H, *m*), 6.16 (1H, *d*, *J* = 5.9 Hz), 6.38 (2H, *s*), 6.49 (1H, *s*). $^{13}$C NMR ($CDCl_3$, 100 MHz), δ: 21.3 ($CH_3$), 55.4 ($2OCH_3$), 76.8 (CH), 100.0 (CH), 106.6 (2CH), 141.3 (C), 160.9 (2C), 170.0 (C).

#### 3.1.5.4 1'-acetoxy-4-methoxychavicol (20)

To a solution of vinylmagnesium bromide freshly prepared from magnesium turning (2.14 g, 89.2 mmol), 1M vinyl bromide (37.0 ml, 37.0 mmol) and catalytic amount of iodine crystal in dry diethyl ether (50.0 ml), a solution of aldehyde **12** (1.68 g, 12.3 mmol) in diethyl ether (10.0 ml) under ice bath was added. The ice bath was removed and the mixture was allowed to warm to room temperature. After the reaction mixture was stored for 3 hrs at room temperature, saturated aqueous $NH_4Cl$ (10.0 ml) solution was added. The mixture was extracted with diethyl ether (2 x 30.0 ml). The resulting organic extracts were combined and solvent was removed under reduced pressure to yield the crude product. The oily product (intermediate **16**) obtained by usual work-up was acetylated with acetic anhydride (1.51 ml, 16.0 mmol) and DMAP (3.09 g, 25.3 mmol) in

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dichloromethane (25.0 ml) in an ice bath. After consumption of starting material and product formation, the reaction was quenched with saturated aqueous $NH_4Cl$ (10.0 ml) solution and extracted with dichloromethane (3 x 15.0 ml), dried ($NaSO_4$) and filtered. Evaporation of filtrate gave a yellow oil which was purified by column chromatography on silica gel, eluting with hexane/ethyl acetate (95:5) afforded the desired product as a yellowish oil **20** (1.57 g, 62%). The spectroscopic summary of analogue **20**: A yellowish oil, Calculated for $C_{12}H_{14}O_3$, 206.0948; Found 43, 65, 77, 91, 104, 121, 131,149, 164, 206. IR (neat) $\upsilon_{max}$, $cm^{-1}$: 1737, 1515, 1236. UV $\lambda_{max}$, nm: 304.5. $^1$H NMR ($CDCl_3$, 400 MHz), δ: 2.07 (3H, *s*), 3.78 (3H, *s*), 5.28 (2H, *dd*, *J* = 10.4 Hz), 5.99 (1H, *m*), 6.22 (1H, *d*, *J* = 5.80 Hz), 6.88 (2H, *d*, *J* = 6.80 Hz), 7.28 (2H, *d*, *J* = 6.80 Hz). $^{13}$C NMR ($CDCl_3$, 100 MHz), δ: 21.3 ($CH_3$), 55.3 ($OCH_3$), 76.3 (CH), 113.9 (2CH), 116.5 ($CH_2$), 128.8 (2CH), 136.4 (CH), 159.5 (C), 169.1 (C).

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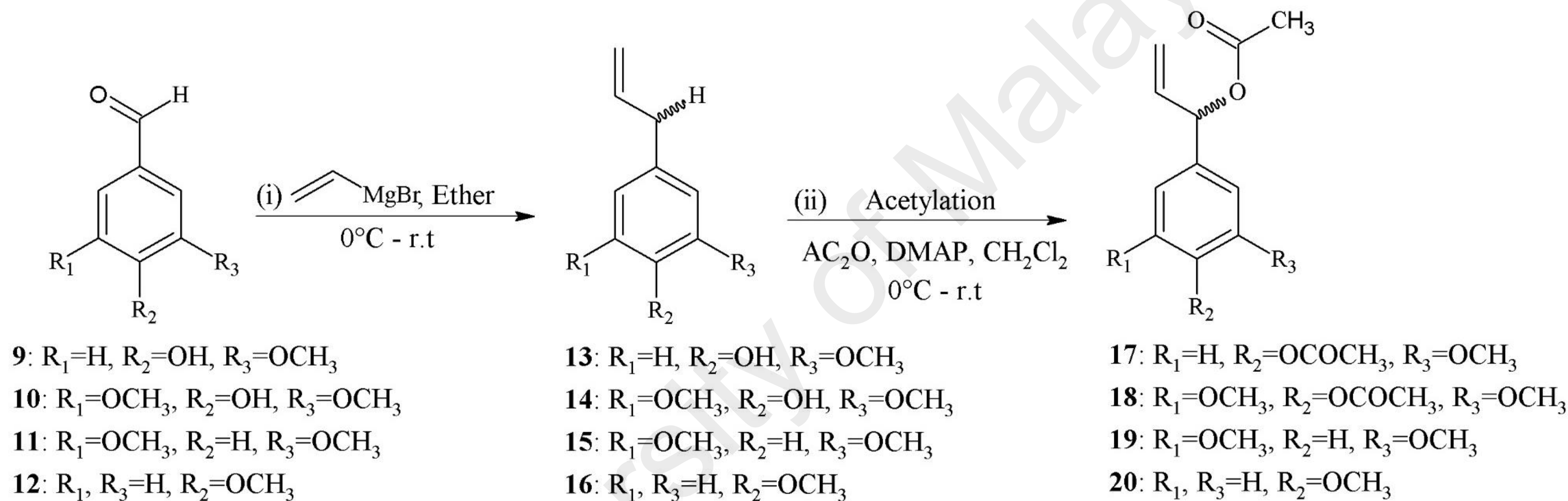


**Figure 3.2:** Schematic preparation of compounds **17-20**.

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### 3.1.6 Preparation of ACA and Its Analogue Solutions

Purified ACA and its hemi-synthetic analogue compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare both stock and working solutions. For preparation of 20 x ACA stock solution, 46.9 mg of pure ACA was dissolved in 10.0 ml of DMSO (Merck, Germany) to get a final concentration of 20.0 mM. The solution was then vortexed vigorously to allow complete dissolution of ACA in DMSO solvent. By performing a 2 x dilution of the ACA stock solution with DMSO, 1 x ACA working solutions with final concentration of 10.0 mM were prepared. The stock solution was stored at 4°C while working solutions were kept at 25°C. To avoid the cytotoxic effects of DMSO, ACA working solution was diluted with the growth medium to a final DMSO concentration that did not exceed 1.0% (v/v). The stock and working solutions of all ACA analogues were prepared based on the similar methods mentioned above.

## 3.2 Cell Lines

### 3.2.1 Cell Lines and Culture Conditions

A total of seven cancer cell lines and one normal cell line were used in this study, which are summarised in the Table 3.1. All cancer cell lines were maintained in cell culture flask containing either RPMI-1640 (Thermo Scientific, USA) or DMEM (Thermo Scientific, USA) medium supplemented with 10.0% (v/v) FBS (Sigma-Aldrich, USA), 100.0 U/ml penicillin (Lonza, USA) and 100.0 µg/ml streptomycin (Lonza, USA). HMEC cells acting as normal cell controls were cultured in serum free MEGM medium (Lonza, USA). All cells were grown as monolayers in a humidified incubator (Memmert, Germany) at 37°C with 5.0% $CO_2$ and 95.0% air.

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**Table 3.1:** The sources and culture media used for cultivation of various human cancer and normal cell lines used in this study.

| Cell Lines | Type | Source | Culture Media |
|---|---|---|---|
| MDA-MB-231 | Human Breast Adenocarcinoma Cells | ATCC | RPMI-1640 |
| MCF-7 | | ATCC | RPMI-1640 |
| RT-112 | Human Bladder Carcinoma Cells | German Collection of Microorganisms and Cell Cultures | RPMI-1640 |
| EJ-28 | | ATCC | RPMI-1640 |
| PC-3 | Human Prostate Adenocarcinoma Cells | ATCC | RPMI-1640 |
| HSC-4 | Human Oral Squamous Carcinoma Cells | CARIF | DMEM |
| HepG2 | Human Hepatocyte Liver Carcinoma Cells | ATCC | DMEM |
| HMEC | Human Mammary Epithelial Cells (Normal Cells) | Lonza, USA | MEGM |

### 3.2.2 Subculturing Monolayer Cell Culture

All cell lines were sub-cultured every two to three days, or when the confluency of the cell in flask reached 80.0-90.0%. The spent cell culture medium was removed discarded using a serological pipette. Cells were rinsed with 1 x PBS (Lonza, USA) to remove any residual traces from medium that can inactivate the trypsin. The PBS solution was removed and 3.0 ml of 0.25% (v/v) trypsin (Lonza, USA)-EDTA (Gibco, USA) solution was added to the culture flask. To ensure complete cells detachment, cell culture flask was incubated at 37°C for 10 mins. Once the cells were detached, equal number of growth medium supplemented with FBS was added to the cell suspension to inactivate the trypsin activity. All the suspension was pipetted into a labelled 15.0 ml centrifuge tube and centrifuged at 1,500 rpm for 10 mins using Centrifuge 5702 (Eppendorf, Germany). The

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supernatant was then discarded and the cell pellet was re-suspended in fresh cell culture medium. For routine cell maintenance, the cells were split into three or four sterile flasks.

### 3.2.3 Cryopreservation of Cell Culture

Prior to cryopreservation of cell lines, cells were grown to 80.0% confluency and checked for its health status. The confluent cells were harvested as described in section 3.2.2. After centrifugation, the cell pellet was collected and re-suspended with a mixture of fresh growth medium with 20.0% (v/v) FBS and 10.0% (v/v) DMSO. DMSO acted as a cryoprotectant. Several stocks of 1.0 ml of aliquots were dispensed into 2.0 ml cryopreservation vial and frozen at -20°C for 12 hrs before transferred to -196°C liquid nitrogen tank.

### 3.2.4 Thawing of Cryopreserved Cells

After removed from nitrogen tank, cryopreserved cells were thawed in 37°C water bath for 5 mins. Each 1.0 ml of thawed cell suspension was diluted with 9.0 ml of cell culture medium containing 10.0% (v/v) FBS and centrifuged at 1,500 rpm for 5 mins. The supernatant containing the cryoprotectant DMSO was discarded and the pellet was re-suspended with prepared medium containing 10.0% (v/v) FBS and 100.0 µg/ml streptomycin. The cells were then maintained at 37°C in a 95.0% humidified incubator circulated with 5.0% of $CO_2$.

### 3.2.5 Cell Counting

The number of viable cells present in a specific population was determined via a dye exclusion viability assay using a haemocytometer (Resistance, Germany). Cells were harvested and re-suspended in growth medium gently. 20.0 µl of cell suspension was aliquoted into a microcentrifuge tube and mixed with 20.0 µl of 0.08% trypan blue

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(Merck, Germany) dye solution. The tube was allowed to stand for about 3 mins. 10.0 µl of the mixture was then pipetted onto a haemocytometer counting chamber, and spread evenly by capillary action. The counting of unstained viable cells in each of the four-square grid corners was performed under the inverted microscope (Nikon, Japan) at 100 x magnification. Each square grid represents a 0.1 $mm^3$ or $10^{-4}$ ml volume and the concentration of cell suspension was determined using Equation 3.1 with a dilution factor of two. The haemocytometer and cover slip were sprayed and cleaned immediately with 70.0% (v/v) ethanol (Thermo Scientific, USA) between samples and after use.

$$\text{Cell concentration (cells/ml)} = \frac{\text{Average Number of Cells Counted}}{\text{Volume counted}} \times \text{Dilution Factor}$$

(Equation 3.1)

## 3.3 Cytotoxicity Assay

### 3.3.1 Preparation of MTT Reagent

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagents were prepared by adding 60.0 mg of MTT (Calbiochem, USA) to 12.0 ml of 1 x PBS (Lonza, USA). To ensure the MTT powder was completely dissolved, the reagent was vigorously shaken and vortexed. MTT working solutions were stored in the dark at room temperature (25°C) and at 4°C in the dark for long storage. The final concentration of the MTT working solution used in MTT assay was 5.0 mg/ml.

### 3.3.2 MTT Assay

The anti-proliferative effects of ACA and its analogues were assessed by measuring changes in cell viability using the MTT assay *in vitro*. The MTT assay is a colorimetric assay that depends on the reduction of yellow MTT dye into insoluble formazan purple

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crystal by viable cells. In brief, both cancer and normal cells were washed with 1x PBS (Lonza, USA) without calcium and magnesium. The PBS solution was aspirated, and cells were detached by covering with 0.25% (v/v) trypsin (Lonza, USA)-EDTA (Gibco, USA) solution. After 5-10 mins of incubation in 37°C, the harvested cells were transferred to the 15.0 ml centrifuge tube with addition of FBS-containing growth media. Cell pellets that formed after centrifugation at 1,500 rpm for 10 mins were re-suspended in media to make a single cell suspension. Determination of number of viable cells was done using the trypan blue exclusion method according to section 3.2.5, to give the desired density of 5.0 x $10^5$ cells/ml. Cells were seeded in triplicates on flat-bottom 96-well plates at 100.0 µl/well with 1.0 x $10^4$ cells and incubated at 37°C overnight to allow for cell attachment to the well surface. After the incubation, cells were treated with increasing concentrations (5.0 to 50.0 µM) of ACA and its analogues for 24 hrs. Wells containing cell culture media alone were served as negative controls and DMSO as solvent controls to ensure that cytotoxicity was not solvent induced. Wells containing cells in gradient concentration (10,000 cells, 5,000 cells, 2,500 cells, 1,250 cells and 0 cells) were used to plot standard curve for quantification purposes. At the end of incubation period, 20.0 µl of 5.0 mg/ml MTT reagent (Calbiochem, USA) was added to all the wells and incubated in the dark at 37°C for 2 hrs until the presence of purple insoluble-formazan precipitate. The contents from the plate were replaced with 200.0 µl of DMSO and agitated on a plate shaker in the dark for 15 mins to allow complete changes of formazan crystals to coloured solution. The absorbance of the solution was measured at 570 nm wavelength with a 650 nm reference wavelength using the Tecan Sunrise® microtiter plate reader (Tecan, Switzerland). The results were then quantified using the Magellan Version 7.1 (Tecan, Switzerland) software.

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## 3.4 Proteasome Inhibition Assay

### 3.4.1 Preparation of Epoxomicin

Epoxomicin, the commercial proteasome inhibitor was used in the proteasome activity assay. For preparation of stock solution, 72.0 µl of DMSO was added to 20.0 µg solid in the provided vial of epoxomicin (Enzo, USA), to produce a final concentration of 500.0 µM. The solution was then vortexed well to ensure that epoxomicin had completely dissolved in DMSO solvent. The stock solution was stored at -20°C for long term usage up to 3 months. Different working concentrations can be prepared by taking 0.04, 0.4, 4.0, 40.0, 400.0 µl of 500.0 µM stock solution and diluted to a final volume of 1.0 ml which resulted in 0.02, 0.2, 2.0, 20.0, 200.0 µM solutions respectively. For further proteasome activity analysis, 5.0 µl of different working solutions was diluted in total volume of 50.0 µl with HEPES buffer (Gibco, USA) or culture medium, to prepare final concentrations of 0.001, 0.01, 0.10, 1.0, 10.0 µM of epoxomicin compound.

### 3.4.2 Preparation of Proteasome-Glo™ Reagent

The enzyme-based and cell-based proteasome inhibition assays were done by using Proteasome-Glo™ 3-Substrate System (Promega, USA) and Proteasome-Glo™ Cell-Based 3-Substrate System (Promega, USA) respectively. Each assay was carried out as indicated in the manufacturer's protocol. Briefly, thawed Proteasome-Glo™ Buffer, lyophilised Luciferin Detection Reagent and three substrates were equilibrated to room temperature in the dark. Once at room temperature the Luciferin Detection Reagent was reconstituted with 10.0 ml of Proteasome-Glo™ Buffer in an amber bottle. Next, the Proteasome-Glo™ Substrate was vortexed well and added to the re-suspended Luciferin Detection Reagent to prepare the Proteasome-Glo™ Reagent. The substrates with appropriate volume used for the chymotrypsin-like, trypsin-like and caspase-like activities were Suc-LLVY-Glo™ Substrate (50.0 µl), Z-LRR-Glo™ Substrate (100.0 µl),

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Z-nLPnLD-Glo™ Substrate (50.0 µl) respectively. For cell-based trypsin-like assay only, 15.0 µl of Inhibitor 1 and 100.0 µl of Inhibitor Mix 2 were added to the substrate-luciferin mixture to reduce non-specific protease activities. To allow removal of any free aminoluciferin and reduction of background luminescence, the prepared Proteasome-Glo™ Reagent was then mixed gently and stored at room temperature in the dark for 60 mins before use. The reagents were added to assay plates, resulting in proteasome cleavage of the luminogenic substrate and generation of aminoluciferin, which is transformed by luciferase to produce "glow-type" luminescence (Figure 3.3). The luminescent signal is directly proportional to the proteasome activity.

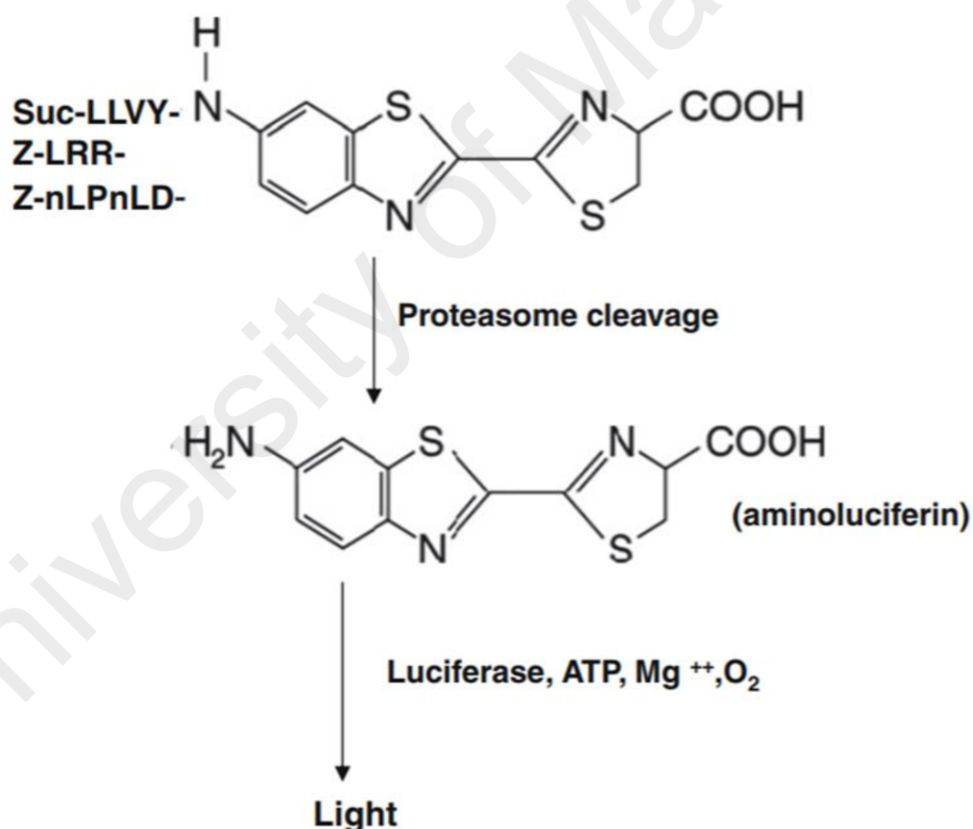


**Figure 3.3:** The reaction of luminogenic, aminoluciferin substrates succeeding proteasome cleavage. Suc-LLVY-, Z-LRR- or Z-nLPnLD-aminoluciferase are specific substrates of the chymotrypsin-like, trypsin-like and caspase-like proteasome active sites, respectively. Following proteasome cleavage, aminoluciferin is released, allowing the luciferase reaction to produce light (Reproduced with permission from Moravec et al., 2009).

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### 3.4.3 Enzyme-Based Proteasome Activity Assay

A total of 5.0 µl of 1.0 mg/ml purified human 20S proteasome (Enzo, USA) was diluted in 1M HEPES buffer (Gibco) to a final volume of 45.0 µl. The diluted 20S proteasome was then dispensed into the white-walled 96-well plate (Enzo, USA). Next, 5.0 µl of ACA and its analogues with different working concentrations were added to the buffer, that is, the 20S proteasome was treated with ACA analogues in a final concentration range of 5.0 to 200.0 µM. Wells containing the commercial proteasome inhibitor epoxomicin (0.001 to 10.0 µM) were tested as inhibitor controls, DMSO was used as solvent controls and well without any 20S proteasome were served as blank. 50.0 µl of Proteasome-Glo™ Reagent was added to each well of the 96-well plate. The plate was left to stand for 5 mins on plate shaker in the dark and then incubated at room temperature for 60 mins. Three proteasome peptidase activities were detected as the relative light unit (RLU) generated from the cleaved substrates in the reagent. Luminescence generated from each reaction was monitored with Synergy H1 Hybrid Multi-Mode Reader (Biotek, USA). The background value was subtracted from each sample.

### 3.4.4 Cell-Based Proteasome Activity Assay

A number of $1.0 \times 10^5$ cells/ml of MDA-MB-231 cells were plated into white 96-well plate (Enzo, USA). Each well was topped up to 50.0 µl with RPMI-1640 growth medium. The plate was incubated overnight in the 5% $CO_2$ humidified incubator at 37°C. After the incubation, existing medium was replaced with 50.0µl of fresh medium before treatment. The cells were treated with increasing concentrations (5.0 to 200.0 µM) of ACA and its analogues for 24 hrs. A well-known proteasome inhibitor epoxomicin was used as inhibitor control, DMSO as solvent control and medium-only well (without cells) served as blank control. 50.0 µl of Proteasome-Glo™ Reagent was added to each well of the 96-



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well plate containing blank, control or test sample. The contents of wells were agitated on plate shaker in the dark for 5 mins. The plate was then incubated at room temperature for 60 mins, after which the luminescence signals were measured in the Synergy H1 Hybrid Multi-Mode Reader (Biotek, USA). The luminescence reading of the blank was subtracted from all samples.

## 3.5 *In silico* Docking

### 3.5.1 Preparation of 20S Proteasome Protein File

The crystal structures of the β1, β2 and β5 subunits of the 20S proteasome were obtained from the yeast 20S proteasome (PDB ID: 1JD2) (Figure 3.4), which is similar to the human proteasome. The β1, β2 and β5 subunits were extracted from the H, N and K chain of 20S proteasome PDB respectively by using Discovery Studio Visualizer 3.1 (Accelrys, Inc, USA). The three separate files were then saved as pdb files for further usage in docking analysis.

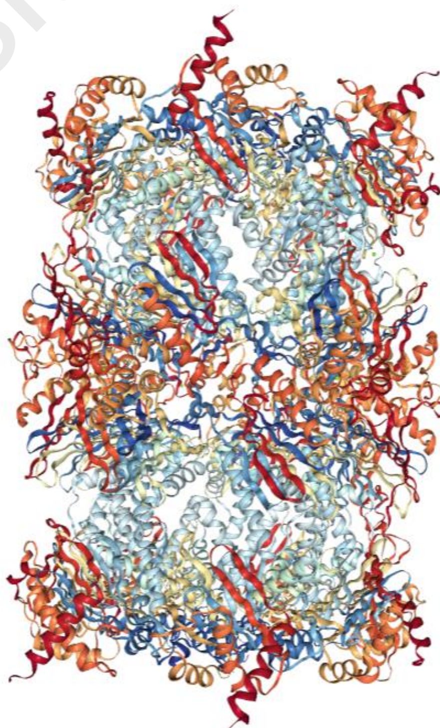

**Figure 3.4:** 3D structure of 20S Proteasome (1JD2).

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### 3.5.2 Preparation of Test Compounds Ligand File

Three dimensional structural models of ACA, AEA and AMCA were drawn using ChemSketch program Version 1.1 (Advanced Chemistry Development, Inc., Canada). The 3D structures of these compounds were saved in mol2 format. The final ligand files were saved in pdbqt format by using AutoDockTools version 1.54 software (The Scripps Research Institute, USA).

### 3.5.3 Preparation of Grid Parameter File (gpf)

The 20S proteasome protein file and the selected test compound ligand file were opened together in AutoDockTools version 1.54 software. A grid box was created by adjusting the defaults value to cover the entire protein. The spacing was set to a default value of 0.375, and the file extension was set as ".gpf". Gpf files were created for other ligands as well. The same process was repeated for all 20S proteasome β subunit protein structures.

### 3.5.4 Preparation of the Docking Parameter File (dpf)

The protein file and the selected ligand file were opened together in AutoDockTools 1.5.2 software. The docking parameters were set as number of GA runs = 100, number of individuals in population = 150 and maximum number of energy minimisation (medium) = 2500000. Cluster tolerance (rmstol) value was set as 2.0 and Lamarckian genetic algorithm was used. The file extension was set as ".dpf". Dpf files were created for other ligands as well. The same process was repeated for all 20S proteasome β subunit protein structures.

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### 3.5.5 Running Autodock

Running autodock using the Autodock 4.0 software (The Scripps Research Institute, USA) requires a protein file, a ligand file, a gpf and a dpf file. Autogrid was run using the command line, autogrid4 –pfilename.gpf –l filename.glg &. Autogrid was followed by autodock using the commandline autodock4 –p filename.dpf –l filename.dlg &. The process was repeated for each protein and each ligand.

### 3.5.6 Docking Analysis

Ligand - protein interactions were analysed using Ligplot 4.4.2 software. The compounds which showed high affinity for the 20S proteasome were identified.

## 3.6 Apoptosis Assay

### 3.6.1 DNA Fragmentation Assay

DNA fragmentation assay by using the Suicide Track$^{TM}$ DNA Ladder Isolation Kit (Calbiochem, USA) was applied for detection of apoptosis induced by ACA and its analogues. MDA-MB-231 breast cancer cells were cultured until 80.0% confluence, followed by treatment with ACA, AEA and AMCA at their $IC_{50}$ concentrations for 24 hrs. The treated cells were then trypsinised and centrifuged at 1,500 rpm for 10 mins. The cell pellet was gently re-suspended in 55.0 µl of Solution #1 (Calbiochem, USA), to allow lysis of cell samples and the inactivation of nucleases. It was then mixed with 20.0 µl of Solution #2 (Calbiochem, USA) and incubated at 37°C to degrade RNA in the cell lysate. In order to isolate DNA from the cell lysate, 25.0 µl of Solution #3 (Calbiochem, USA) was added, followed by overnight incubation at 50°C. Next day, 500.0 µl of resuspension buffer (Calbiochem, USA) was added and mixed well by pipetting. Subsequently, 2.0 µl of a fluorescent dye, Pellet Paint Co-Precipitant (Calbiochem USA) and 60.0 µl of sodium acetate (3M, pH 5.2) (Calbiochem, USA) were added to the mixture, for better



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visualisation of the DNA pellet. A total of 662.0 µl of isopropanol (Merck, Germany) was added to the mixture and centrifuged at 16,000 x *g* for 5 mins. The supernatant was removed with a pipette tip, leaving behind a pink DNA pellet in the bottom of the tube. The DNA pellet was rinsed twice with 500.0 µl of 70.0% (v/v) ethanol (Merck, Germany) and 100.0% (v/v) ethanol (Merck, Germany) respectively, accompanied with centrifugation at 16,000 x *g* for 5 mins between each rinsing step. All DNA pellets were air-dried at room temperature for 15 mins and re-suspended in 50.0 µl of resuspension buffer (Calbiochem, USA). The DNA samples were kept at -20°C for further use.

### 3.6.2 Determination of DNA Concentration

The concentration and purification of extracted DNA samples were determined using the NanoDrop 2000 (Thermo Scientific, USA). The concentration of each DNA sample was diluted 100-fold, by adding 495.0 µl of distilled water to 5.0 µl of DNA sample. Before commencing DNA quantification, 1.0 µl of distilled water was pipetted to the bottom pedestal and Nanodrop software was initiated. The top and bottom pedestals were then wiped with lint-free Kim-wipes (Kimberly-Clark, Canada). An aliquot of 1.0 µl of resuspension buffer was then placed onto the bottom pedestal by pipette. A blank reading was taken to compare with the extracted DNA samples. 1.0 µl of DNA sample was then transferred to the bottom pedestal and the quantity of DNA present was measured at an absorbance of 260 nm. Evaluation of DNA purity was performed by studying the absorbance ratios for A260/A280 and A 260/A230. Before conducting the agarose gel electrophoresis, the DNA samples were normalised with resuspension buffer to ensure consistency in sample concentration.

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### 3.6.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyse DNA fragments from cells treated with ACA and its analogues. Firstly, a 1.0% (w/v) agarose gel was made by dissolving 0.5 g of agarose powder in 50.0 ml of 1 x TAE buffer (Amresco, USA). The agarose was then melted in the microwave oven (Panasonic, Malaysia) for 2 mins. The molten agarose was poured into a 5.5 x 12.0 cm gel cassette (BayGen, China), an 8-well comb (BayGen, China) with 1.5 mm spacing was inserted in the gel cassette and the agarose solution was allowed to set for approximately 45 mins at room temperature. Once the agarose gel had set, the casting tray was placed into an electrophoresis chamber containing 1x TAE buffer after careful removal of the well comb. DNA was prepared for loading by mixing 10.0 µl of extracted DNA with 2.0 µl of 6 x loading dye [10.0 mM Tris-HCl (pH7.6), 0.03% (w/v) bromophenol blue, 0.03% (v/v) xylene cyanol, 60.0% (v/v) glycerol, 60.0 mM EDTA] (Calbiochem, USA). 5.0 µl of the 100 bp DNA ladder (Ready-to-use) (Calbiochem, USA) was loaded into lane 1 of the gel and 12.0 µl of extracted DNA/loading buffer was loaded into each subsequent well. The running tank was connected to a power pack and electrophoresis was carried out at 120.0 V and 80.0 mA for 1 hr. After completion of electrophoresis, the gel was stained with 0.2 µg/l of ethidium bromide (Promega, USA) for 20 mins and de-stained in distilled water for 5 mins. The DNA was visualised under UV transillumination and images were captured using ChemiImagerTM 4400 (Alpha Innotech, USA) at 302 nm wavelength.

## 3.7 Migration Assay

### 3.7.1 Wound Healing Assay

The anti-migration effects of ACA and its analogues were determined using the wound-healing assay. MDA-MB-231 cells were seeded in six-well plates and grown to 80.0% confluence. After an overnight cultivation, complete medium was then replaced



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by serum-free medium treated with 1.0 μg/ml Mitomycin-C (Calbiochem, USA), and the cells were further incubated at 37°C for 2 hrs to stop cell proliferation. A vertical scratch was made using a sterile pipette tip across the well, and cell debris was removed by washing with 1 x PBS twice. Cells were treated with control solvent or ACA and its analogues at their 20.0% inhibitory concentrations ($IC_{20}$) (Appendix E) in serum-free medium for 24 hrs at 37°C. $IC_{20}$ were used instead of $IC_{50}$, because $IC_{20}$ were less toxic on the cells. Images of wounded cells were captured using an inverted fluorescence microscope, Nikon Eclipse TS-100 (Nikon, Japan), at 0 and 24 hrs after wounding. The distance between two sides of the wound was analysed using Tscratch software, version 1.0 (MathWorks, USA). The cell migration rate was calculated using the formulas shown in Equation 3.2.

$$\text{Wound healing (\%)} = \frac{\text{(open image area at start - open image area at end)}}{\text{open image area at start}} \times 100$$

(Equation 3.2)

## 3.8 Protein Expression Analysis

### 3.8.1 Extraction of Cytoplasmic and Nuclear Proteins

Non-denatured, active nuclear and cytoplasmic proteins were extracted from all treated cancer cell lines. Following treatment, the spent media with detached cells were aspirated from the 6-well plates and collected in the 15.0 ml centrifuge tubes. The remaining cells were then detached using 0.25% (v/v) trypsin (Lonza, USA)-EDTA (Gibco, USA) and mixed with the cells from the spent media. Cells were then spun down at 1,500 rpm for 10 mins and the supernatant was removed. The nuclear and cytoplasmic protein fractionation was performed using the NE-PER® nuclear and cytoplasmic extraction kit (Pierce, USA) according to manufacturer’s instructions. The cell pellet was re-suspended in ice cold CER I (Pierce, USA) at a volume of 200.0 μl containing 1 x



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protease/phosphatase inhibitor cocktail (Pierce, USA). The sample was vortexed vigorously for 15 secs, and subsequently incubated for 10 mins on ice to allow complete lysis of cells. Next, 11.0 µl of CER II (Pierce, USA) was added, and the tube vortexed for 10 secs. The sample was incubated on ice for 2 mins, and then vortexed again for 10 secs. After centrifugation for 5 mins at 4°C and 16,000 x *g* using the Sorvall Legend Micro 17R (Thermo Scientific, USA) refrigerated centrifuge, the supernatant containing the cytoplasmic extract was immediately transferred to a clean pre-chilled tube. The pellet, which contains intact nuclei, was re-suspended in 100.0 µl of ice cold NER (Pierce, USA), and then vortexed for 15 secs. The sample was placed in ice while continuing to vortex for 15 secs every 10 mins, for a total duration of 40 mins. Next, the sample was centrifuged for 10 mins at 4°C and 16,000 x *g* and the supernatant containing the nuclear extract was immediately transferred to a new pre-chilled tube.

### 3.8.2 BCA Protein Quantification

Prior to use, protein concentration was determined using the Pierce$^{TM}$ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, USA). This assay is well suited for the quantification of total protein content even in samples that contain up to 5.0% detergents by using colorimetric detection method (Smith et al., 1985). The BCA working reagent, which contains BCA and cupric sulphate was prepared freshly by mixing reagent A and reagent B in a 50:1 ratio and mixed until light green in colour. The working reagent was added to each sample of unknown concentration in PCR tube with a ratio of 8:1. Each sample was mixed by pipetting and then centrifuged briefly. Samples were incubated at 37°C for 30 mins and cooled to room temperature for 10 mins. Absorbance of each sample was measured at 562 nm wavelength using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). A standard curve was prepared using a standard dilution series of Bovine Serum Albumin (BSA) at concentrations of 100 µg/ml

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to 1,500 µg/ml. Protein concentrations of samples were determined from the graph. Both cytoplasmic and nuclear protein concentrations were normalised with distilled water to a final concentration of 3.0 mg/ml and 2.0 mg/ml respectively, before conducting protein electrophoresis.

### 3.8.3 Protein Sample Preparation

A mixture of the normalised cytoplasmic and nuclear proteins in a 1:2 ratio was prepared. Then, 5.0 µl of 4 x non-reducing Pierce$^{TM}$ LDS Sample Loading Buffer (Thermo Scientific, USA) was added to the 20.0 µl of the protein mixture. All samples were boiled in a dry bath incubator (Allsheng, China) at 95°C for 5 mins and allowed to cool to room temperature. A total of 20.0 µl of each protein sample was loaded into each well for SDS-PAGE.

### 3.8.4 SDS-PAGE

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to fractionate extracted proteins based upon their molecular size. 12.0% (w/v) and 7.5% (w/v) resolving gels were used to separate proteins ranging in size between 14.0 to 70.0 kDa and 24.0 to 205.0 kDa, respectively. The appropriate resolving gel and 4.0% (w/v) stacking gel were prepared by mixing the reagents listed in Table 3.2, with TEMED and freshly prepared APS being added last to initiate gel polymerisation. The resolving gel was loaded into the gap between the glass plates (Bio-Rad, USA) in the assembled gel sandwich and left to polymerise for 45 mins. 0.1% (v/v) SDS (Promega, USA) was immediately laid over the resolving gel to prevent oxidisation and dehydration of the gel. When polymerisation was completed, the overlaid 0.1% SDS solution was completely removed. 4.0% stacking gel was then added on top of the polymerised resolving gel until 100.0% of the glass plate was filled. A 10-well comb with a 0.75 mm thickness was then

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inserted into the stacking gel without trapping any bubbles. After the polymerisation of the gels was completed, the gel comb was removed. The glass plate sandwich was then transferred to a Mini-PROTEN® 3 Cell gel tank (Bio-Rad, USA). 1x TGS running buffer (Bio-Rad, USA) was added into the tank and each well was flushed with this buffer to remove any traces of unpolymerised gel. 5.0 µl of Biotinylated Protein Ladder (Cell Signaling, USA) and 20.0 µl of denatured protein samples were loaded into the wells in a pre-determined order (Protein ladder sizes are listed in Appendix B). Gel electrophoresis was performed by running the gel at 110.0 V with free-flowing current for 15 mins using Power Supply-PowerPac (Bio-Rad, USA) until the samples reached the border to the resolving gel. The SDS-PAGE was completed when the bromophenol blue band has left the resolving gel which occurred approximately after further 60 mins applying a voltage of 150.0 V with free-flowing current to the chamber.

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**Table 3.2:** List of reagents used for the preparation of 4.0% stacking gel, 7.5% and 12.5% resolving gel for SDS-PAGE.

| Reagents | Stacking Gel (4.0%) | Resolving Gel | |
|---|---|---|---|
| | | 7.5% (24-205 kDa) | 12.0% (14-70 kDa) |
| 40.0% (w/v) Acrylamide/Bis-acrylamide (Bio-Rad, USA) | 500.0 µl | 2.82 ml | 4.50 ml |
| 0.5M Tris-HCl (pH6.8) (Promega, USA) | 1.26 ml | - | - |
| 1.5M Tris-HCl (pH8.8) (Promega, USA) | - | 3.75 ml | 3.75 ml |
| 10.0% (w/v) SDS (Promega, USA) | 50.0 µl | 150 µl | 150 µl |
| $dH_2O$ | 3.18 ml | 8.20 ml | 6.52 ml |
| TEMED (Acros, USA) | 5.0 µl | 7.50 µl | 7.50 µl |
| 10.0% (w/v) APS (Pierce, USA) | 25.0 µl | 75.0 µl | 75.0 µl |
| 1.0% (w/v) Bromophenol Blue (Thermo Fisher, USA) | 10.0 µl | - | - |
| **Total Volume** | **5.0 ml** | **15.0 ml** | **15.0 ml** |

### 3.8.5 Western Blot

Upon completion of electrophoresis, the gel was carefully removed from the glass plate sandwich and cut into the right size. The electrophoresed gel was sandwiched by two pieces of Extra Thick Blot Paper (Bio-Rad, USA) and one piece of 0.2 µm pore size nitrocellulose transfer membrane (Bio-Rad, USA). Prior to assembly in the transfer apparatus, they were immersed in 1 x TGS transfer buffer (Bio-Rad, USA) with either 10.0% (for large proteins) or 20.0% (for small proteins) (v/v) methanol (Merck,

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Germany) for 10 mins. A transfer sandwich was then placed in between two carbon plates (cathode and anode) in the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, USA). During the assembly any trapped air bubbles were removed using a blotting roller (Milipore, USA). The transfer apparatus was connected to the MP-2AP Power Supply (Major Science, Taiwan) set at 50.0 mA with free-flowing voltage for 90 mins. Once proteins were transferred onto nitrocellulose membrane, it subsequently was visualised by staining with Ponceau S Stain which consisting of 0.1% (w/v) Ponceau S (Sigma, USA) and 5.0% (v/v) acetic acid (Merck, Germany) for 5 mins. Then, the membrane was washed twice with $dH_2O$ for 2 mins each. After washing, nitrocellulose membrane was kept in 5.0% (w/v) BSA (Amresco, USA), 1 x TBS buffer and 0.1% (v/v) Tween-20 (Merck, Germany) for 1 hr while shaking on a Mini-Shaker Multi Bio 3D (Biosan, Latvia) in order to block non-specific binding sites. Blocked membrane was then hybridised overnight with the appropriate amount of primary antibody diluted in 10.0 ml of blocking buffer at 4°C. Optimal working dilution of different antibodies was used according to manufacturer's instruction as mentioned in Table 3.3. The blot continued incubated the next day with primary antibody at room temperature for 1 hr with agitation. Post incubation, the membrane was washed three times with 1 x TBST buffer for 5 mins each with agitation. Then the appropriate secondary antibody conjugated to horse radish peroxidase (HRP) conjugated diluted 1:1000 in blocking buffer (Table 3.4) was applied to the membrane and agitated for 1 hr at room temperature. Following incubation, nitrocellulose membrane was washed 3 times with 1 x TBST buffer for 5 mins each, followed by a single wash in 1 x TBS buffer for 5 mins. The membrane was then blotted dry from excess wash buffer by using Kim-wipes. The detection of the HRP conjugated antibody on the membrane was done by subjecting Western Bright ECL HRP substrate (Advansta, USA) for 2 mins in the dark. Protein bands were visualised on a Fusion FX7 imaging system (Vilber Lourmat, Germany). Anti-GAPDH control antibody was used for

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normalisation of band intensities. The intensities of protein bands were quantitated using ImageJ Version 1.48 (National Institutes of Health, USA) densitometry software.

**Table 3.3:** Summary of host species, dilution and antigen molecular weight from Cell Signaling for primary antibodies used in western blot experiments.

| Antibodies | Host Species | Dilution | Antigen Molecular Weight (kDa) |
|---|---|---|---|
| Ubiquitin (PD41) | Mouse | 1:1000 | Broad range |
| PARP | Rabbit | 1:1000 | 116, 89 |
| Bcl-2 | Mouse | 1:1000 | 26 |
| Bcl-xL | Rabbit | 1:1000 | 30 |
| Bax | Rabbit | 1:1000 | 20 |
| p53 | Mouse | 1:1000 | 53 |
| Integrin β1 | Rabbit | 1:1000 | 115,135 |
| FAK | Rabbit | 1:1000 | 125 |
| Phospho-FAK | Rabbit | 1:1000 | 125 |
| Akt | Rabbit | 1:1000 | 60 |
| Phospho-Akt | Rabbit | 1:1000 | 60 |
| GAPDH | Rabbit | 1:1000 | 37 |

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**Table 3.4:** Summary of host species, dilution and targeted primary antibodies from Cell Signaling for secondary antibodies used in western blot experiments.

| Antibodies | Host Species | Dilution | Targeted Primary Antibodies |
|---|---|---|---|
| Anti-mouse IgG-HRP | Horse | 1:1000 | Ubiquitin (PD41), Bcl-2, p53 |
| Anti-rabbit IgG-HRP | Goat | 1:1000 | PARP, Bcl-xL, Bax, Integrin β1, FAK, Phospho-FAK, Akt, Phospho-Akt, GAPDH |

## 3.9 Statistical Analysis

All assays were performed in triplicate independent experiments. The results were presented as mean ± standard error mean (SEM). One-way ANOVA was used to analyse the statistically significant differences with a confidence level of $p \leq 0.05$.

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# CHAPTER 4: RESULTS

## 4.1 ACA and Its Hemi-Synthetic Analogues

### 4.1.1 Hemi-Synthesis of ACA Analogues

The main focus of this study was to chemically synthesis ACA analogues, followed by evaluation of their degree of cytotoxicity, regulation of UPS, apoptotic effects and anti-migratory effects in the treatment of human breast cancer. Isolation and purification of compounds from the dichloromethane ($CH_2Cl_2$) extract yielded purified 1'S-1'-acetoxychavicol acetate, whereas the hemi-synthesis of compounds yielded 4-allyl-2,6-dimethoxyphenol, **2**; 4-allyl anisole, **5**; 4-allyl-2,6-dimethoxyphenyl acetate, **6**; 1-phenyl-2-propen-1-yl acetate, **7**; eugenol acetate, **8**; 1'-acetoxyeugenol acetate (AEA), **17**; 1'-acetoxy-3,5-dimethoxychavicol acetate (AMCA), **18**; 1'-acetoxy-3,5-dimethoxychavicol, **19** and 1'-acetoxy-4-methoxychavicol, **20**. The chemical structures of ACA and its nine analogues are illustrated in Figure 4.1.

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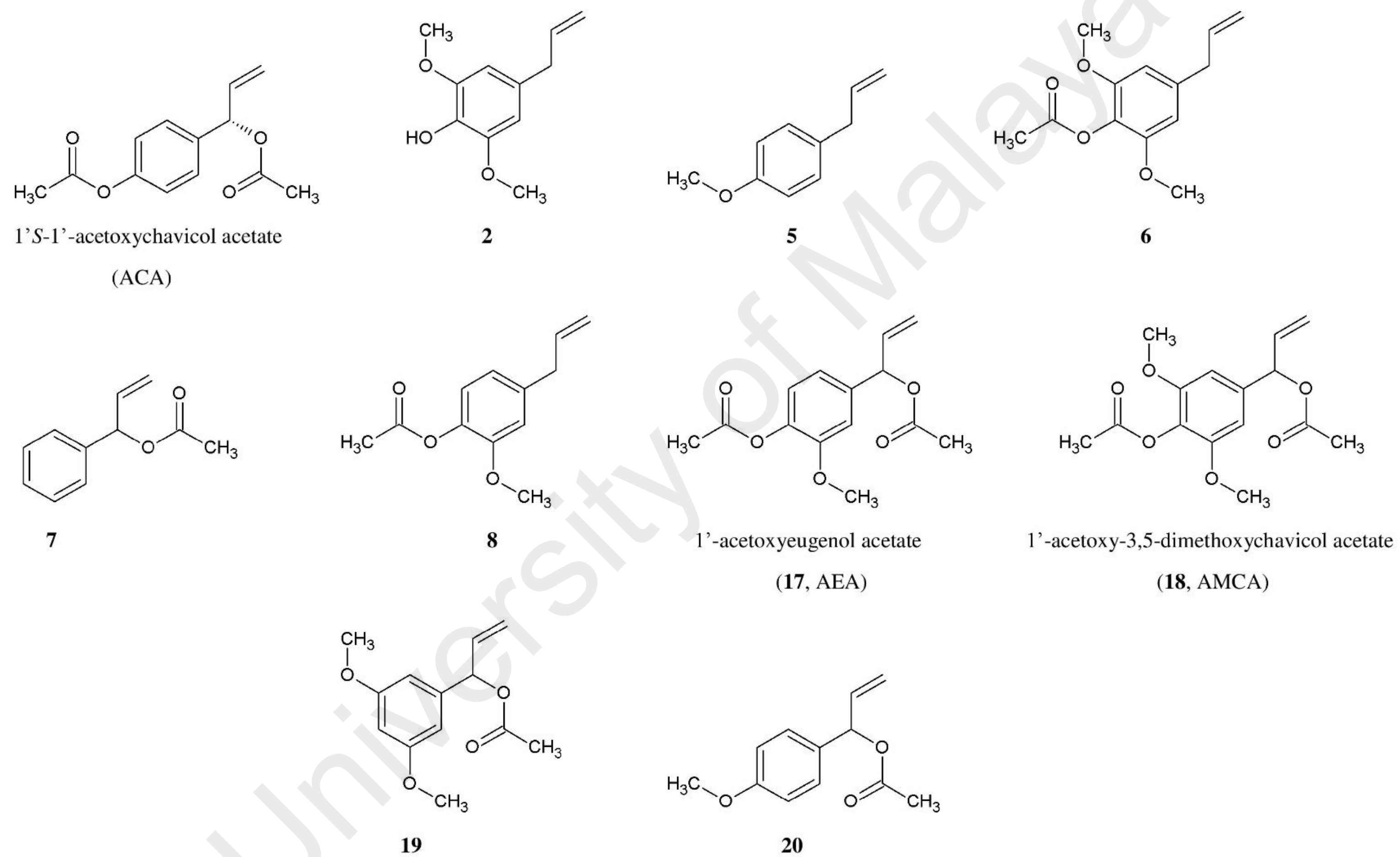


**Figure 4.1:** Chemical structure of ACA and its nine analogues.

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### 4.1.2 Structural Comparison between ACA and Its Analogues

Structural elucidation demonstrated that all compounds have 2'-3' terminal double bonds at the benzene ring. Previous literature has shown that 2'-3' terminal bonds were important for the biological activity (Murakami et al., 2000). ACA possesses a 1'-acetoxy group at the benzene ring, which is also found in AEA, AMCA, analogues **7**, **19** and **20** (Figure 4.2). Besides, AEA, AMCA, analogues **6** and **8** have similarity with ACA in which they have substitution of an acetoxy group at the C4 position (Figure 4.3). In addition, AEA, AMCA, analogues **2**, **6**, **8** and **19** possess methoxy group at the C3 position, which is not present in ACA (Figure 4.4). Furthermore, methoxy group attached at C5 position can be found in AMCA, analogues **2**, **6** and **19** (Figure 4.5). To date, little has been known about chemical properties of ACA analogues in relation to their biological implications in comparison to ACA. Thus, it is crucial to analyse the SAR of ACA and its analogues.

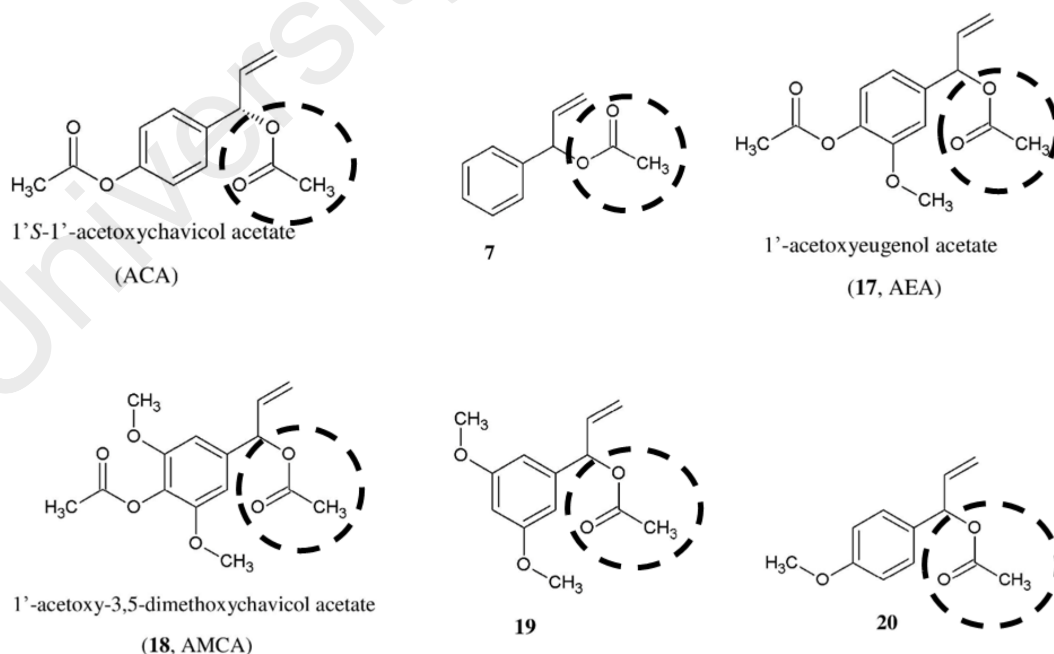


**Figure 4.2:** ACA and its analogues with 1'-acetoxy group.

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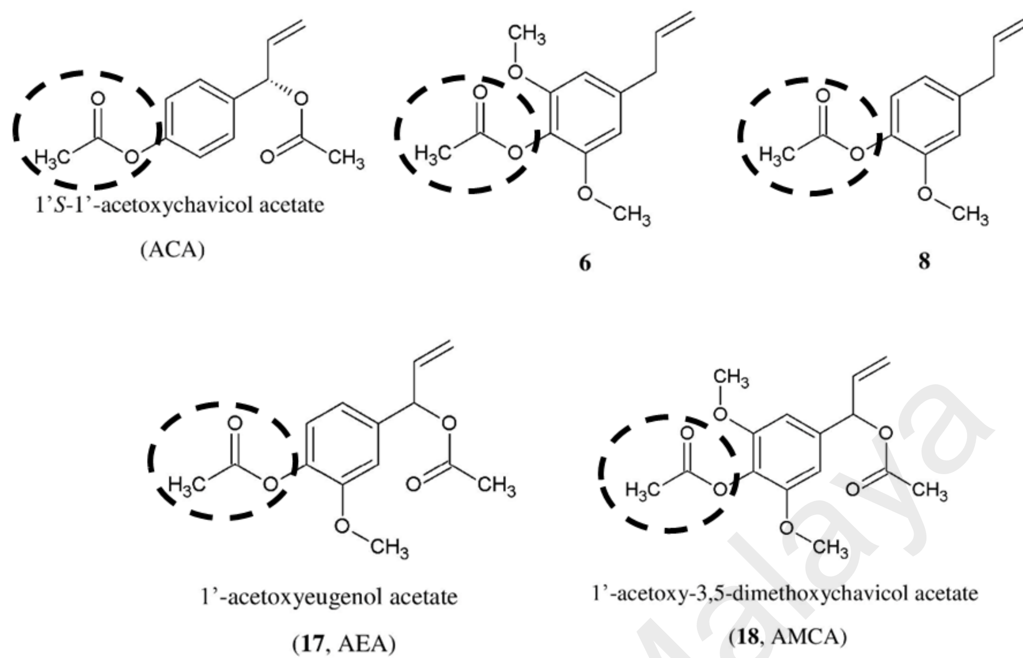


**Figure 4.3:** ACA and its analogues with 4-acetoxy group.

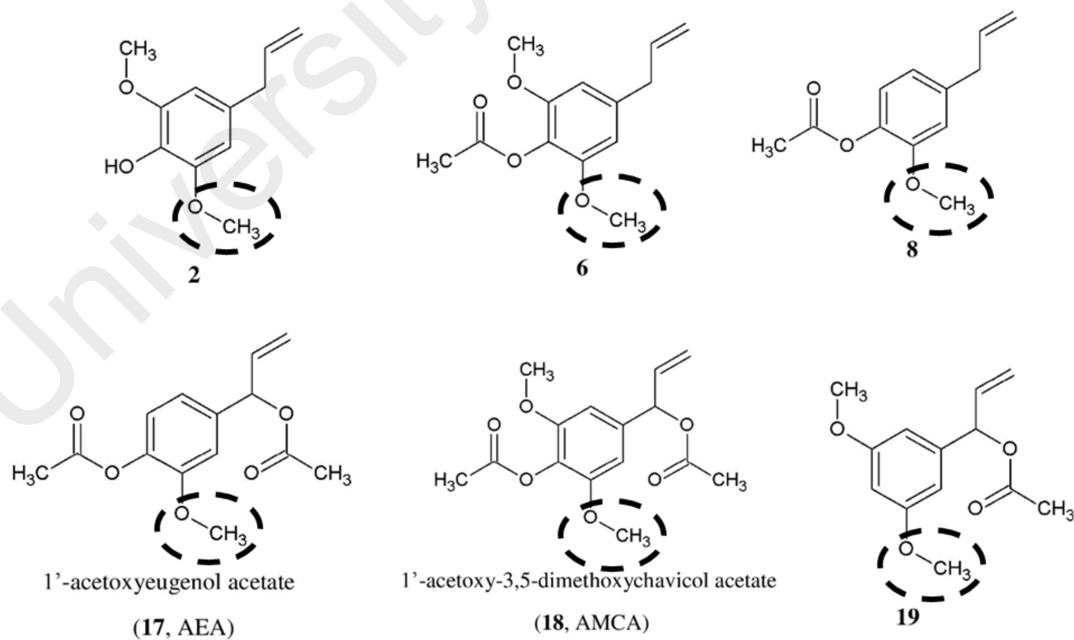


**Figure 4.4:** ACA analogues with 3-methoxy group.

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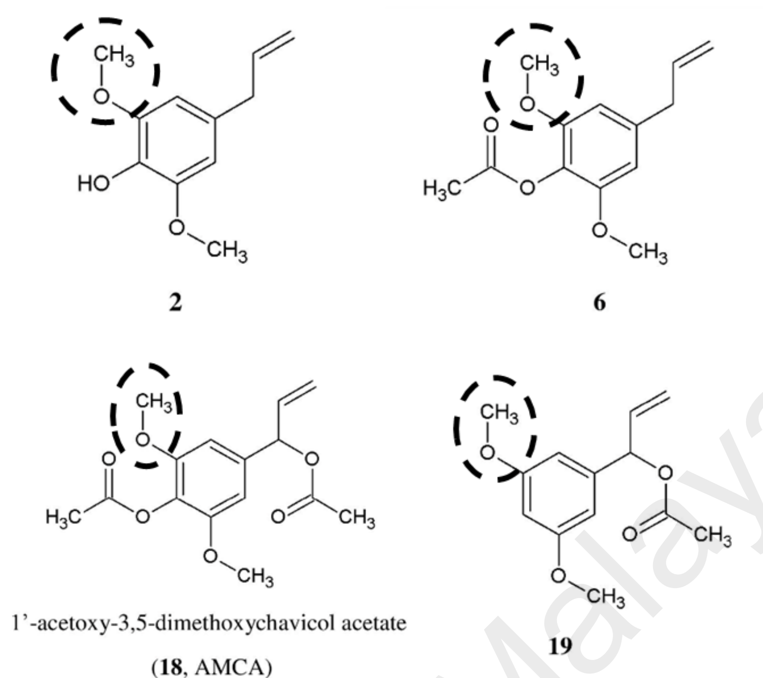


**Figure 4.5:** ACA analogues with 5-methoxy group.

## 4.2 MTT Cytotoxicity Assay

### 4.2.1 Cytotoxic Effects of Various ACA Analogues on Cancer Cell Lines

The MTT assay was used to evaluate the cytotoxicity and anti-proliferative effects of ACA and its nine analogues on seven human cancer cell lines (MDA-MB-231, MCF-7, RT-112, EJ-28, PC-3, HSC-4, HepG2) and one normal human cell line (HMEC). MTT data obtained was also used to determine specific $IC_{50}$ values used in all consecutive experiments. All $IC_{50}$ values were determined based on the concentration of compounds required to kill 50.0 % of the cell population, and are summarised in Table 4.1. The complete MTT cytotoxicity assay data on each individual analogue are shown in Appendix C. All graphs shown are representative of mean values from independent triplicate experiments with ±SEM.

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As shown in Figures 4.6 to 4.12, ACA, AEA and AMCA showed significant biological activity and were able to induce cytotoxicity at least in one cancer cell line with the potencies ≤ 30.0 μM. All other tested analogues indicated weak cytotoxicity with less than 40% killing even after 50.0 μM treatment over 24 hrs. Solvent control with DMSO at ≤ 1.0% (v/v) (shown in Appendix D) showed insignificant effects on cancer cell viability, indicating that cytotoxicity was induced as a result of treatment with tested compounds instead of DMSO, which is known to be cytotoxic at high concentrations. Based on $IC_{50}$ values, the reduction of cellular viability of ACA was found to be greatest in MDA-MB-231, HSC-4, EJ-28, RT-112, HepG2, PC-3, MCF-7 cells in descending order. This indicated that ACA induced cell death most efficiently in MDA-MB-231 breast adenocarcinoma with an $IC_{50}$ value of 4.8 μM. Other $IC_{50}$ values corresponding to different cancer cell lines tested are summarised in Table 4.1. AEA was found to induce high levels of cytotoxicity in bladder adenocarcinoma (EJ-28), hepatocarcinoma (HepG2) and oral squamous carcinoma (HSC-4), while AMCA only showed cytotoxicity effects against breast adenocarcinoma (MDA-MB-231) (Table 4.1). Thus, the MDA-MB-231 cell line was selected for the consecutive assays in order to evaluate the effects of these three cytotoxic analogues.

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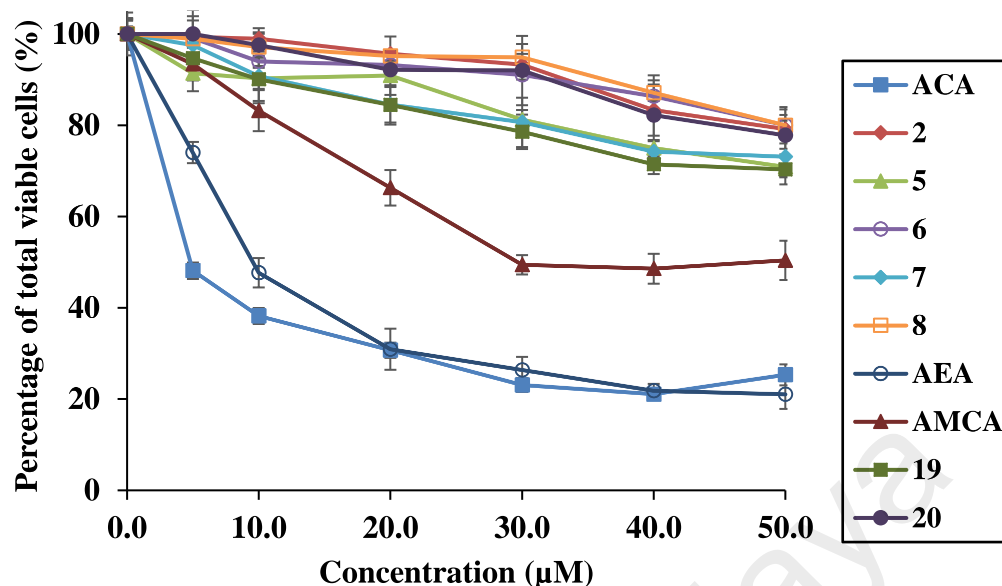


**Figure 4.6:** Comparison of total viable cells on MDA-MB-231 human breast cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. ACA and AEA showed the best cytotoxic effect followed by AMCA in the MDA-MB-231 cells.

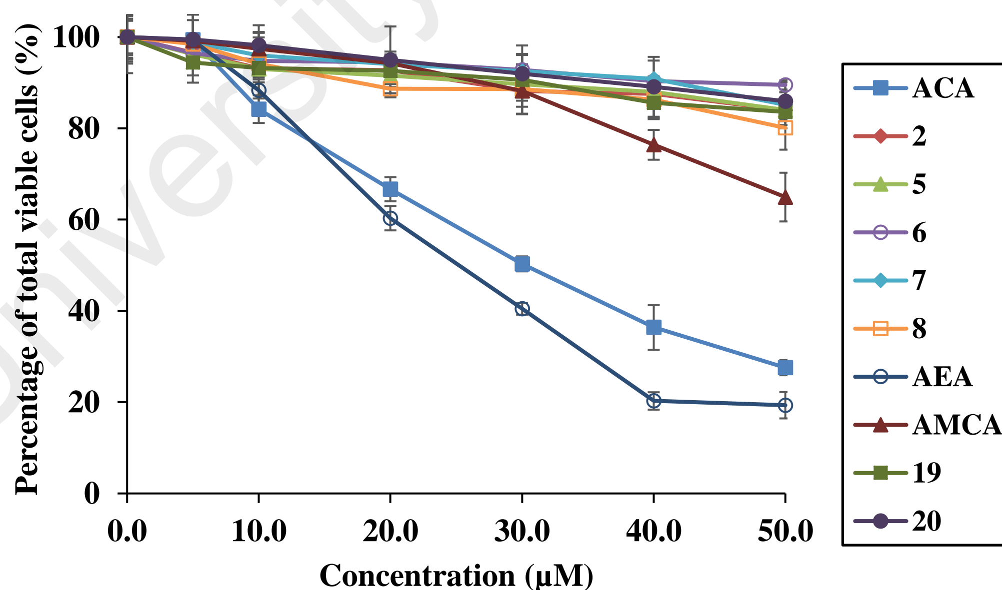


**Figure 4.7:** Comparison of total viable cells on MCF-7 human breast cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. ACA and AEA showed the best cytotoxic effect in MCF-7 cells.

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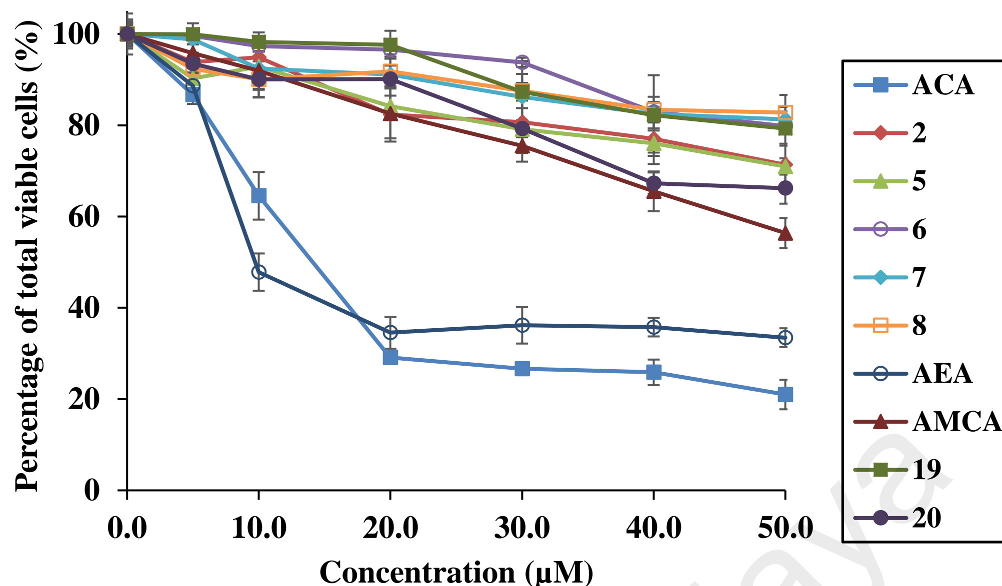


**Figure 4.8:** Comparison of total viable cells on RT-112 human bladder cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. ACA and AEA showed the best cytotoxic effect in RT-112 cells.

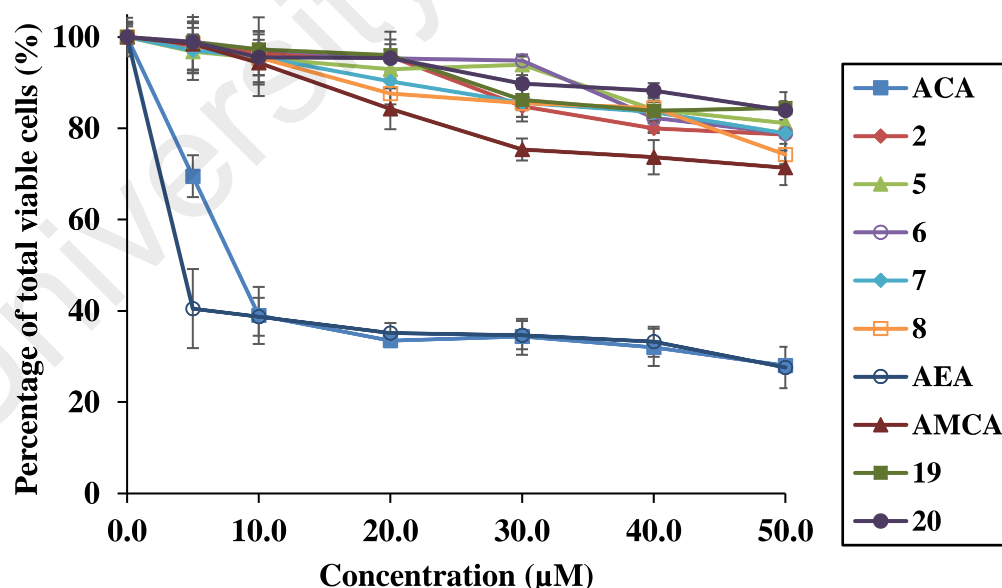


**Figure 4.9:** Comparison of total viable cells on EJ-28 human bladder cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. ACA and AEA showed the best cytotoxic effect in EJ-28 cells.

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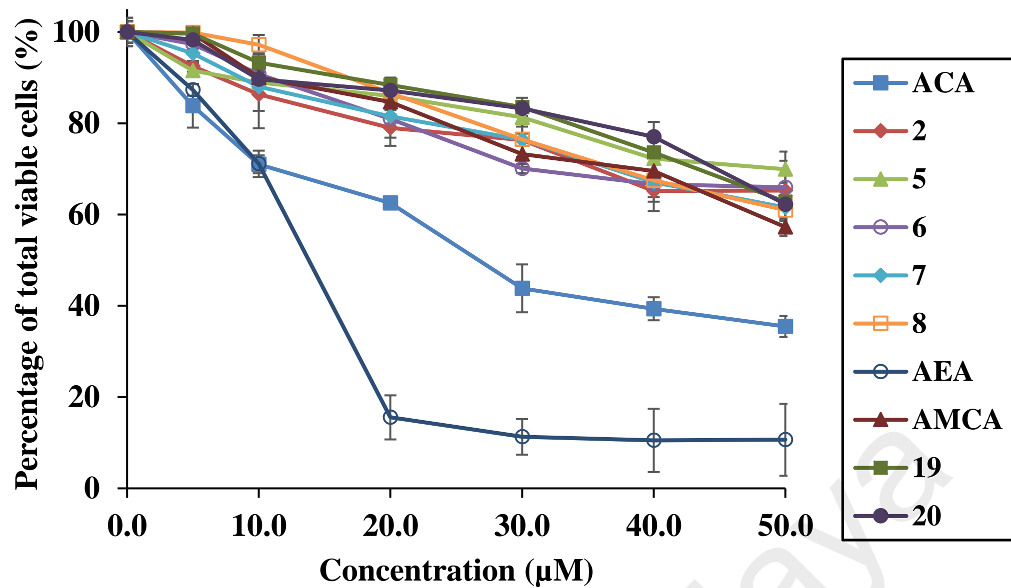


**Figure 4.10:** Comparison of total viable cells on PC-3 human prostate cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. AEA followed by ACA showed the best cytotoxic effect in PC-3 cells.

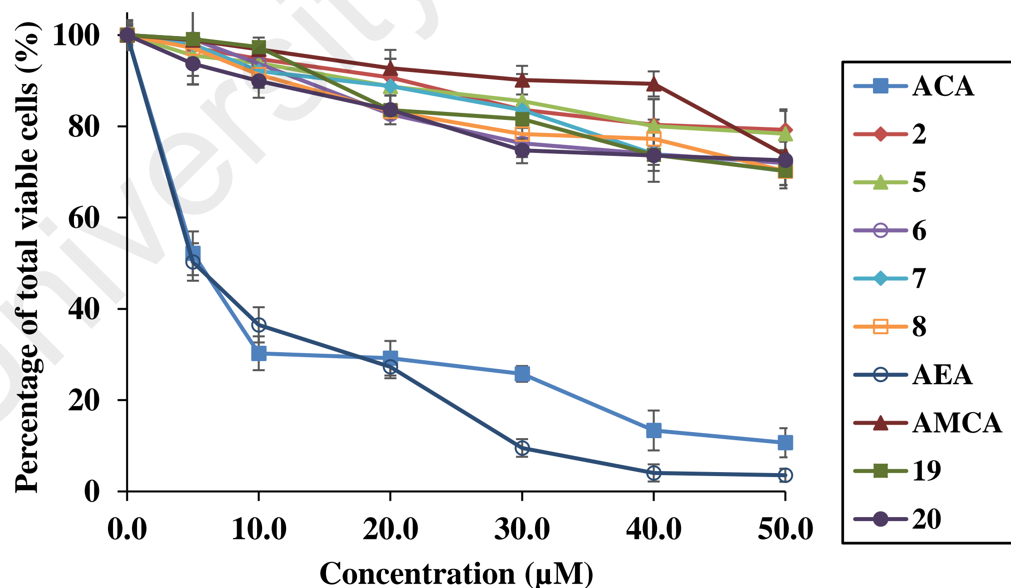


**Figure 4.11:** Comparison of total viable cells on HSC-4 human oral cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. ACA and AEA showed the best cytotoxic effect in HSC-4 cells.

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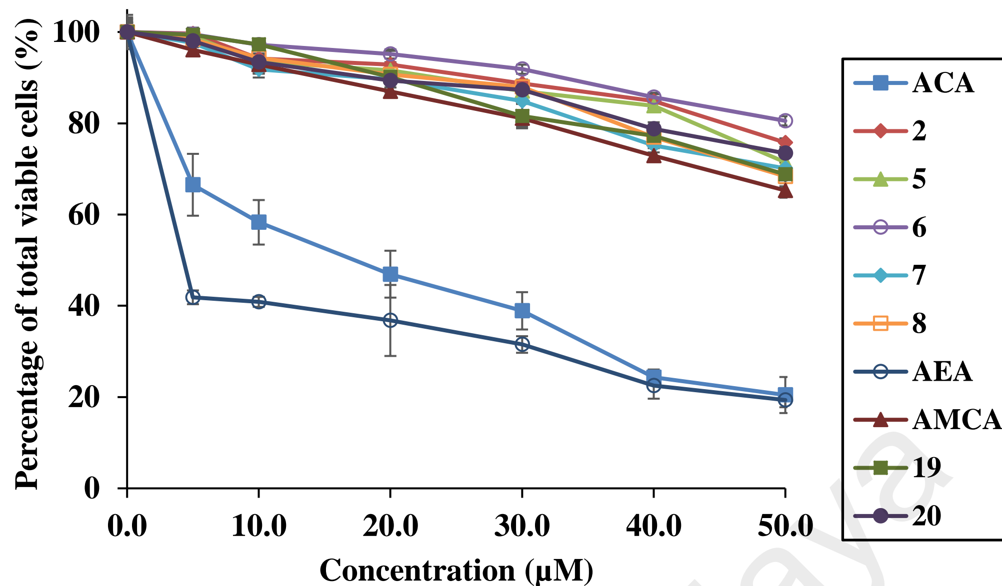


**Figure 4.12:** Comparison of total viable cells on HepG2 human liver cancer cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time. ACA and AEA showed the best cytotoxic effect in HepG2 cells.

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**Table 4.1:** $IC_{50}$ values of ACA and its analogues on various human cancer cell lines as obtained from MTT cytotoxicity assays.

| Cell Lines | $IC_{50}$ (µM)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **ACA** | **2** | **5** | **6** | **7** | **8** | **AEA** | **AMCA** | **19** | **20** |
| Breast adenocarcinoma (MDA-MB-231) | 4.8± 0.4 | N/A (79.2%) | N/A (70.9%) | N/A (80.0%) | N/A (73.1%) | N/A (80.0%) | 9.5± 0.3 | 29.6± 5.6 | N/A (70.3%) | N/A (77.8%) |
| Breast adenocarcinoma (MCF-7) | 30.0± 0.3 | N/A (84.0%) | N/A (84.0%) | N/A (89.5%) | N/A (85.2%) | N/A (80.1%) | 25.2± 0.6 | N/A (64.9%) | N/A (83.6%) | N/A (86.0%) |
| Bladder carcinoma (RT-112) | 14.1± 3.8 | N/A (71.4%) | N/A (71.0%) | N/A (79.8%) | N/A (81.3%) | N/A (82.8%) | 10.9± 2.4 | N/A (56.4%) | N/A (79.3%) | N/A (66.2%) |
| Bladder carcinoma (EJ-28) | 8.2± 0.9 | N/A (78.7%) | N/A (81.2%) | N/A (79.0%) | N/A (78.9%) | N/A (74.3%) | 4.2± 2.2 | N/A (71.4%) | N/A (84.5%) | N/A (83.9%) |
| Prostate carcinoma (PC-3) | 26.7± 2.3 | N/A (65.3%) | N/A (69.9%) | N/A (66.0%) | N/A (61.6%) | N/A (70.0%) | 13.8± 0.8 | N/A (57.3%) | N/A (62.8%) | N/A (62.3%) |
| Oral squamous carcinoma (HSC-4) | 5.5± 0.5 | N/A (79.2%) | N/A (78.4%) | N/A (72.0%) | N/A (70.2%) | N/A (70.2%) | 5.1± 0.2 | N/A (73.7%) | N/A (70.2%) | N/A (72.5%) |
| Hepatocyte carcinoma (HepG2) | 18.0± 0.8 | N/A (75.8%) | N/A (71.5%) | N/A (80.5%) | N/A (70.1%) | N/A (68.4%) | 4.3± 0.5 | N/A (65.3%) | N/A (68.8%) | N/A (73.4%) |
| Human Mammary Epithelial Cells (HMEC) | N/A (79.9%) | N/A (81.1%) | N/A (78.3%) | N/A (78.1%) | N/A (77.7%) | N/A (78.5%) | N/A (79.2%) | N/A (79.7%) | N/A (82.4%) | N/A (79.0%) |

* N/A denotes that $IC_{50}$ values were not attainable within the maximum tested concentration of said analogue, percentage in bracket (%) dictates percentage of cell viability upon maximum treatment with 50.0 µM of each compound.

### 4.2.2 Cytotoxic Effects of ACA Analogues on HMEC Normal Cell Controls

Before conducting further downstream assays, the cytotoxic effects of ACA and its analogues were also assessed on human normal mammary epithelial cells (HMEC). This was to ensure that ACA and its analogues did not induce cytotoxicity in normal cells which is a vital drug screening requirement before their further development as anti-cancer drugs. Figure 4.13 indicated that all compounds were shown to reveal little cytotoxic activity towards HMEC, the normal human breast cells control. Cell viability levels were maintained about 80.0% after 24 hrs treatment at 50.0 µM concentration, indicating non-toxic effects against normal cells at therapeutic doses.

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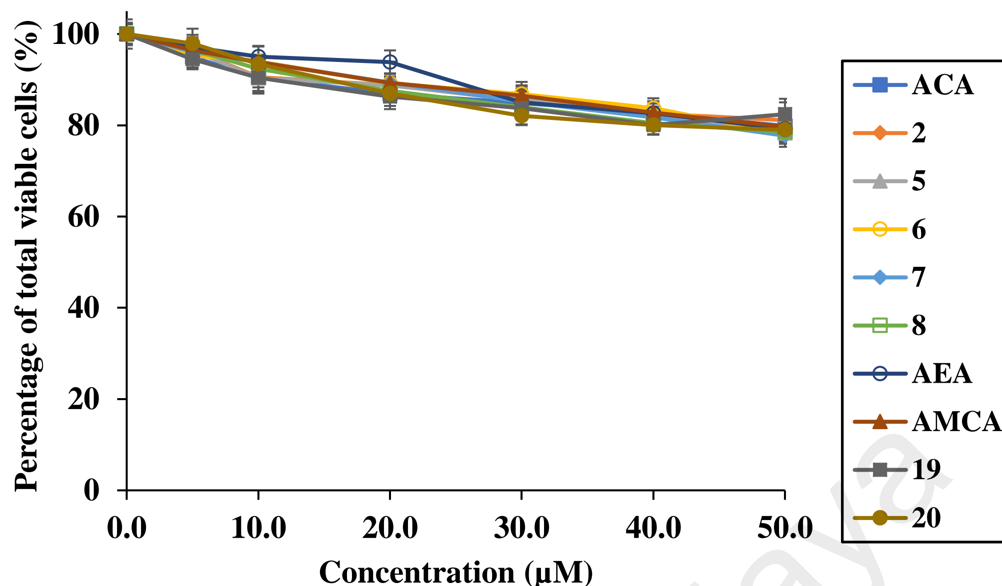


**Figure 4.13:** Comparison of total viable cells on HMEC human mammary epithelial cells after treatment with various ACA analogues at different concentrations (0.0 – 50.0 µM) after 24 hrs post-treatment time.

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### 4.2.3 SAR Analysis on Anti-Proliferative Activity

In order to better understand the SAR of hemi-synthesised ACA, some deductions were drawn from the structural modifications that might have direct or indirect contribution to the anti-cancer effects. In this study, three compounds (ACA, AEA, AMCA possessed 1'-acetoxy group and showed better cytotoxicity than the other analogues. This highlighted the importance of 1'-acetoxy group in governing anti-proliferative activity. Absence of the 1'-acetoxy group (analogues **2**, **5**, **6**, **8**) could lead to a reduction of inhibitory effects on cancer cell growth. Interestingly, AEA exhibited greater potency in MCF-7, HepG2, RT-112, EJ-28, PC-3 cell lines in comparison to ACA, indicating that the addition of a methoxy group at the 3-position displayed a crucial role in governing its anti-proliferative effects. Since analogue **2** possessed a methoxy group at 3-position, a weak cytotoxic effect may have been attributed to the replacement

of the acetoxy group by a hydroxy group at the 4-position. This was further supported by another analogue, compound **19** that had no acetoxy group at the 4-position, with insignificant cytotoxic effects, indicating that the importance of *para*-substitution of the benzene ring with acetoxy group in governing anti-cancer activity. In addition, the replacement of 4-acetoxy group with 4-methoxy (analogues **5** and **20**) reduced the activity. On the other hand, AMCA which possessed an additional methoxy group at the 5-position compared to AEA, exerted a weaker effect against MDA-MB-231 cells, suggesting that the presence of an additional methoxy group at 5-position may reduce the cytotoxic activity. However, AMCA performed better than the other inactive analogues in terms of cytotoxic activity, which suggested the 5-methoxy group is deemed acceptable for the induction of cytotoxicity effects. As shown in Figure 4.14, the recommendations on the anti-proliferative activity are summarised as follows: (1) *Para*-substitution of acetoxy group and 1'-acetoxy group at the benzene ring are compulsory for the activity. (2) Substitution of methoxy group at 3-position may help in anti-proliferation. (3) 4-hydroxy and 4-methoxy groups eliminate the activity. (4) Placement of an additional methoxy group at 5-position weakens the activity.

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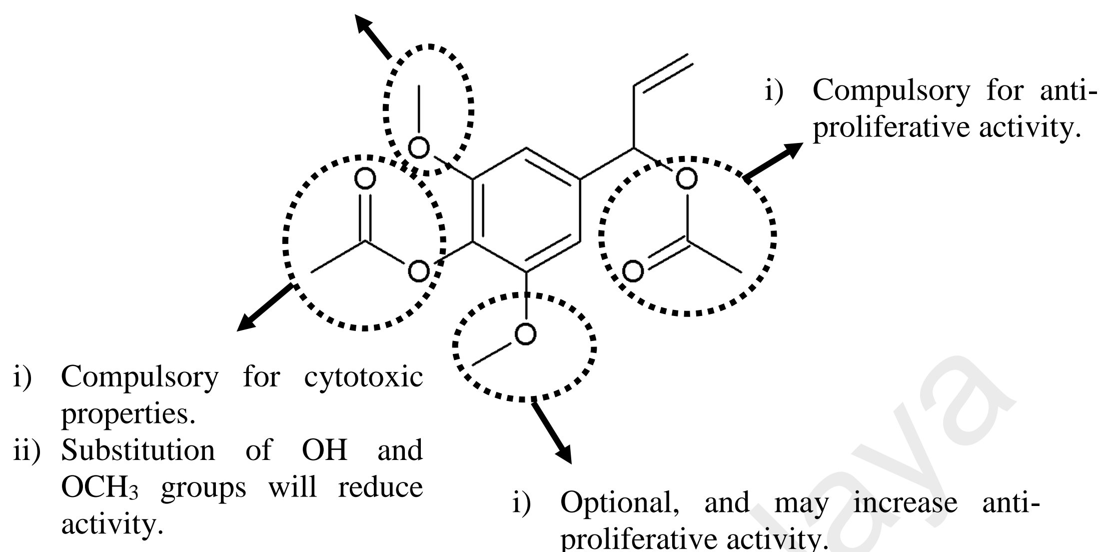


**Figure 4.14:** SAR analysis of individual chemical structure groups within ACA and its analogues towards anti-proliferative properties on various human cancer cell lines.

## 4.3 Ubiquitin-Proteasome System (UPS) Analysis

### 4.3.1 20S Proteasome Activity

To provide direct evidence for the inhibition of 20S proteasome activities by ACA and its analogues, cell-free proteasome activity assay was performed using a purified human 20S proteasome in the presence of tested compound up to 200.0 µM. As shown in the Figures 4.15 – 4.17, ACA and its analogues selectively inhibited the proteasomal peptidase activity, whereby they exerted the best inhibition over the chymotrypsin-like activity among three proteolytic activities. Besides, epoxomicin was used as positive control as it is a potent inhibitor in proteasome activity (Figure 4.18). The chymotrypsin-like activity of the purified 20S proteasome was significantly inhibited by epoxomicin with an $IC_{50}$ value of 0.0095 µM, while ACA and AEA displayed moderate inhibition with the $IC_{50}$ values of 137.0 µM and 199.50 µM, respectively (Table 4.2). Notably,

AMCA did not show any significant proteasomal inhibition in three proteolytic activities. All ACA analogues showed weak inhibition over trypsin-like and caspase-like activities with less than 40% inhibition even after 200.0 µM treatment. On the other hand, epoxomicin significantly reduced these two catalytic activities with $IC_{50}$ values of 1.00 µM and 3.50 µM, respectively. In short, ACA and AEA were revealed to inhibit chymotrypsin-like activity of 20S proteasome, but they were less active than the commercial proteasome inhibitor, epoxomicin.

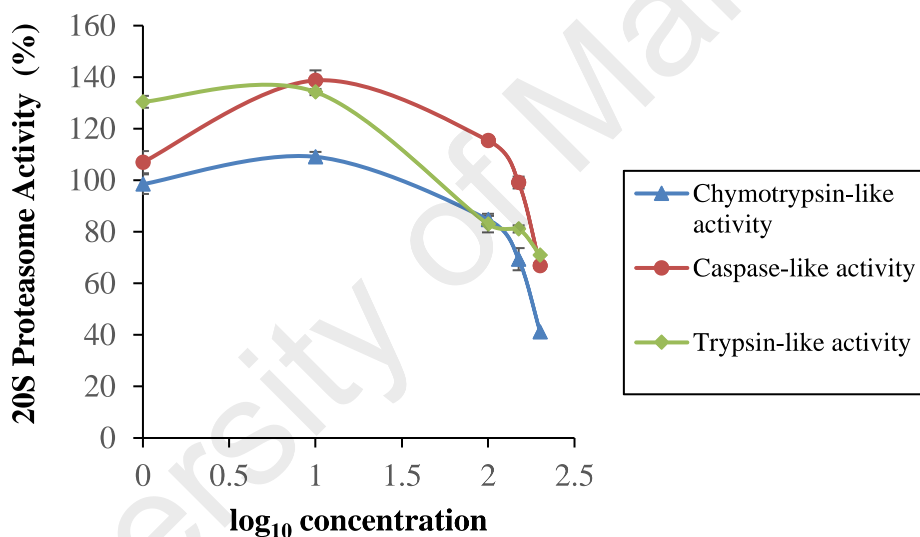


**Figure 4.15:** Comparison of three proteolytic activities of purified 20S proteasome after treatment with ACA at different concentrations (1.0 – 200.0 µM).

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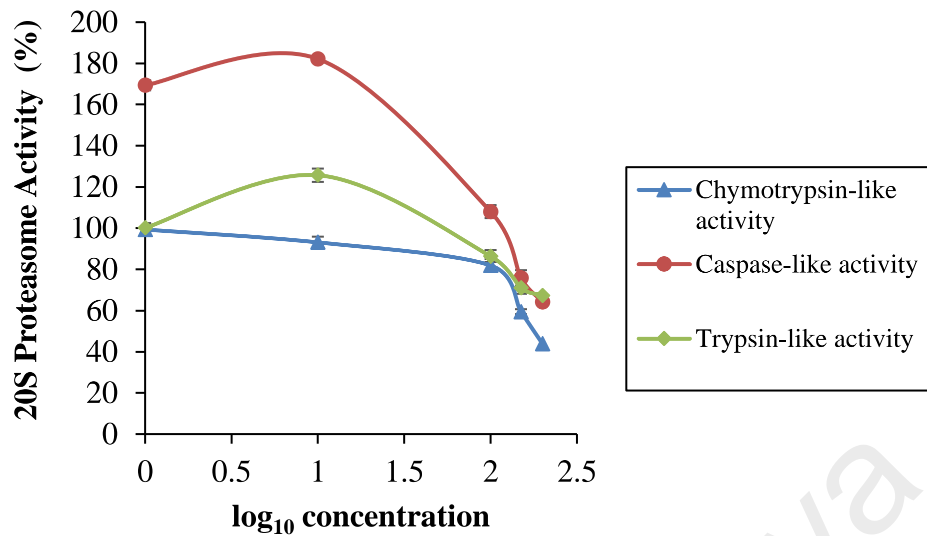


**Figure 4.16:** Comparison of three proteolytic activities of purified 20S proteasome after treatment with AEA at different concentrations (1.0 – 200.0 µM).

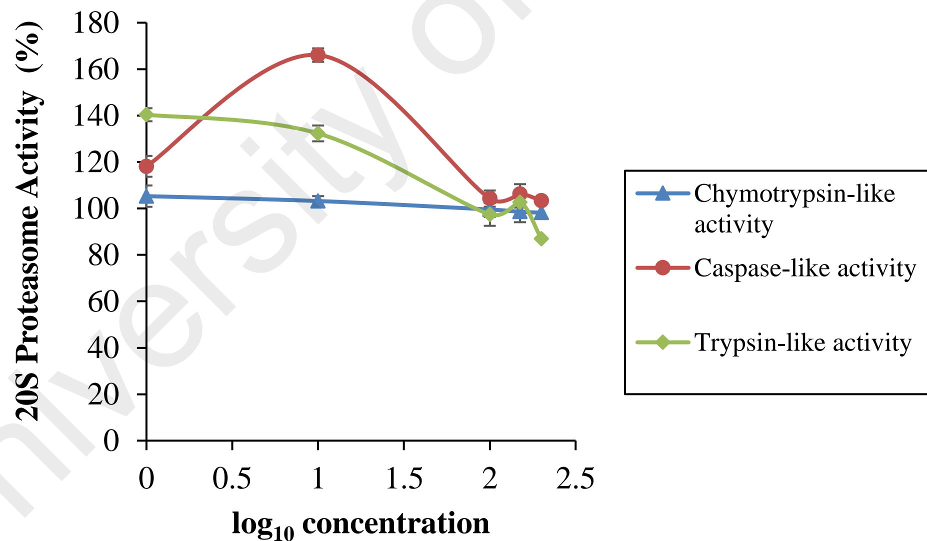


**Figure 4.17:** Comparison of three proteolytic activities of purified 20S proteasome after treatment with AMCA at different concentrations (1.0 – 200.0 µM).

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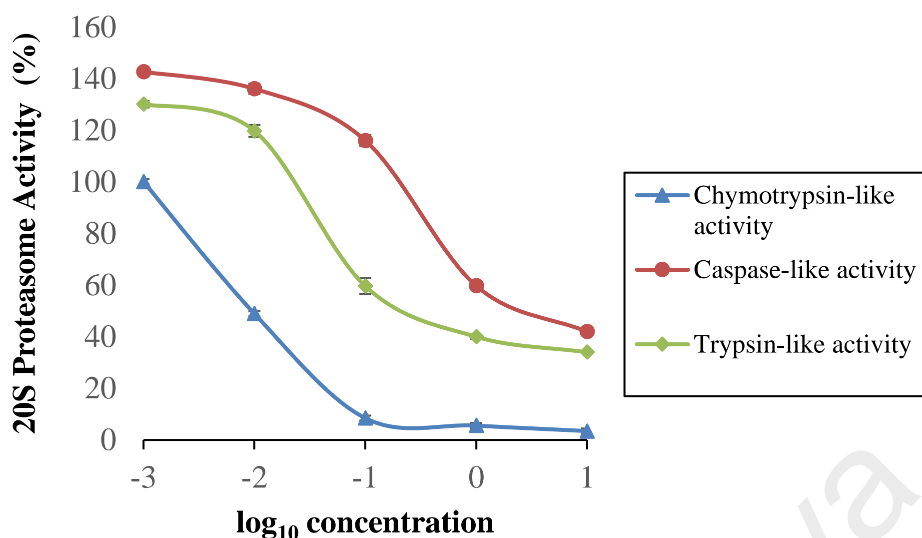


**Figure 4.18:** Comparison of three proteolytic activities of purified 20S proteasome after treatment with epoxomicin at different concentrations (0.001 – 10.0 µM).

**Table 4.2:** $IC_{50}$ values of ACA and its analogues for 20S proteasomal activities.

| **Proteolytic Activities** | **$IC_{50}$ (µM)*** | | | |
|---|---|---|---|---|
| | **ACA** | **AEA** | **AMCA** | **Epoxomicin** |
| Chymotrypsin-like activity | 137.0± 1.3 | 199.5± 5.7 | N/A (98.2%) | 0.0095± 0.037 |
| Trypsin-like activity | N/A (71.0%) | N/A (67.3%) | N/A (86.9%) | 1.00± 0.67 |
| Caspase-like activity | N/A (66.9%) | N/A (64.2%) | N/A (103.4%) | 3.50± 1.23 |

* N/A denotes that $IC_{50}$ values were not attainable within the maximum tested concentration of said analogue, percentage in bracket (%) dictates percentage of proteasome activity upon maximum treatment with 200.0 µM of each compound.

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### 4.3.2 Cellular Proteasome Activity

The effects of ACA and its analogues on inhibition of 26S proteasome in MDA-MB-231 cells were determined by using cellular proteasomal activity assay. The results showed that ACA and its analogues significantly inhibited proteasomal activities in MDA-MB-231 cells except for caspase-like activity (Figures 4.19 - 4.21). As a positive control, epoxomicin was shown to inhibit all three catalytic activities in the cellular proteasome (Figure 4.22). ACA, AEA and AMCA exerted 50.0% inhibition on the cellular proteasomal chymotrypsin-like activity at about 32.5 – 36.2 µM, while on the trypsin-like activity at about 86.8 -111.3 µM (Table 4.3).

Compared with the inhibition on 20S proteasome, it was found that less concentration of the ACA analogues is needed to reach 50% proteasomal inhibition in MDA-MB-231 breast cancer cells. This difference could be due to the stability of ACA analogues in cells and/ or existence of other ACA-binding proteins.

However, there is still a huge difference of the $IC_{50}$ values between ACA, its analogues and epoxomicin, in which the latter was much more effective in the inhibition of the three catalytic activities. In addition, ACA and its analogues did not significantly inhibit the caspase-like activity of cellular proteasome, as only 46% inhibition was achieved even after 200.0 µM treatment.

Taken all together, ACA, AEA and AMCA were shown to inhibit chymotrypsin-like and trypsin-like activities of cellular proteasome in MDA-MB-231 cells, but they were not significantly potent as epoxomicin.

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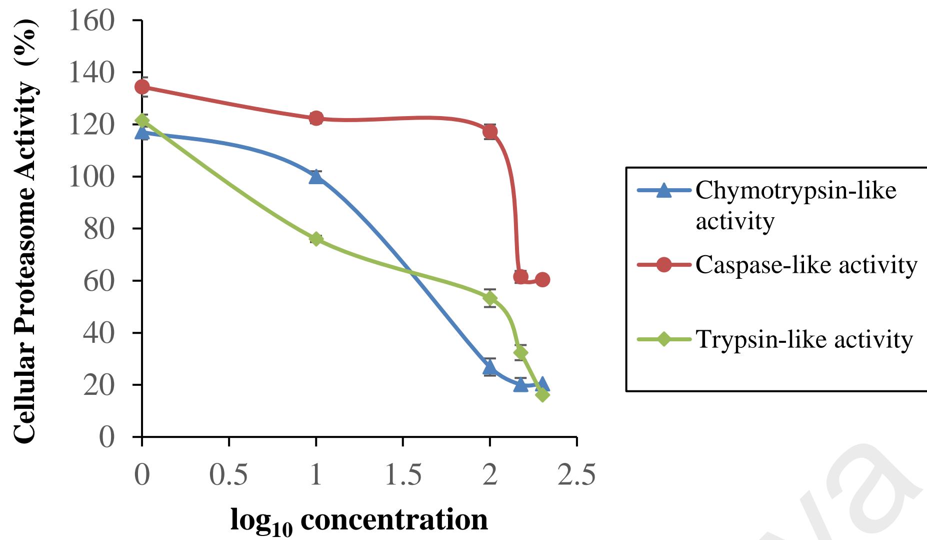


**Figure 4.19:** Comparison of three proteolytic activities of cellular proteasome in MDA-MB-231 cells after treatment with ACA at different concentrations (1.0 – 200.0 µM).

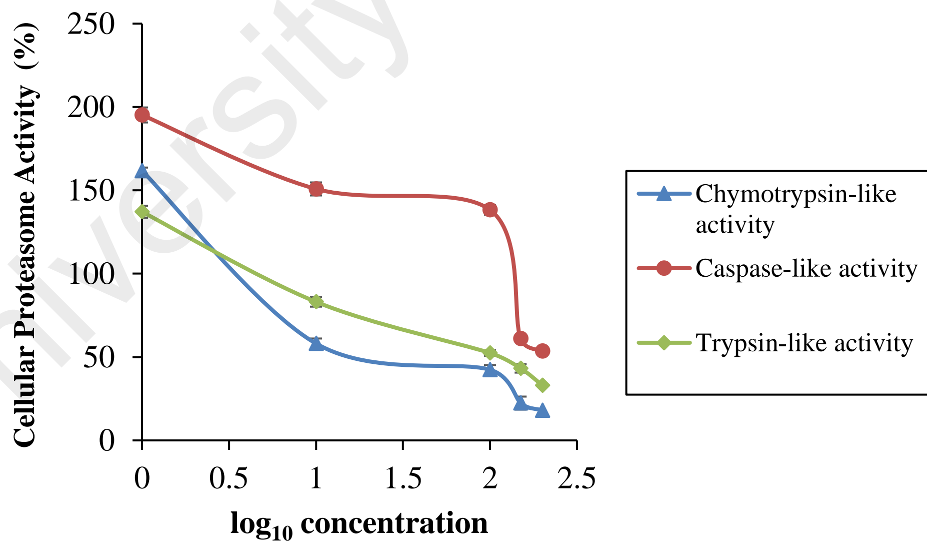


**Figure 4.20:** Comparison of three proteolytic activities of cellular proteasome in MDA-MB-231 cells after treatment with AEA at different concentrations (1.0 – 200.0 µM).

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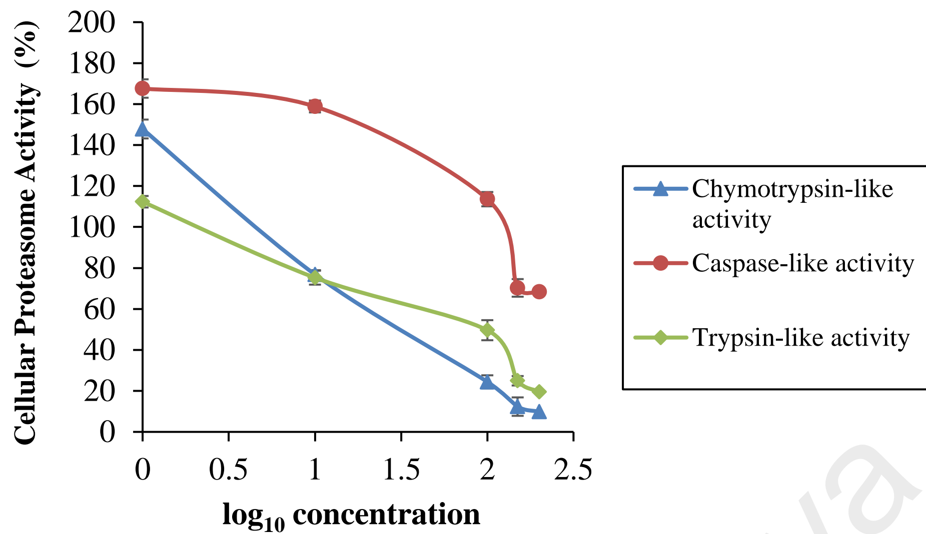


**Figure 4.21:** Comparison of three proteolytic activities of cellular proteasome in MDA-MB-231 cells after treatment with AMCA at different concentrations (1.0 – 200.0 µM).

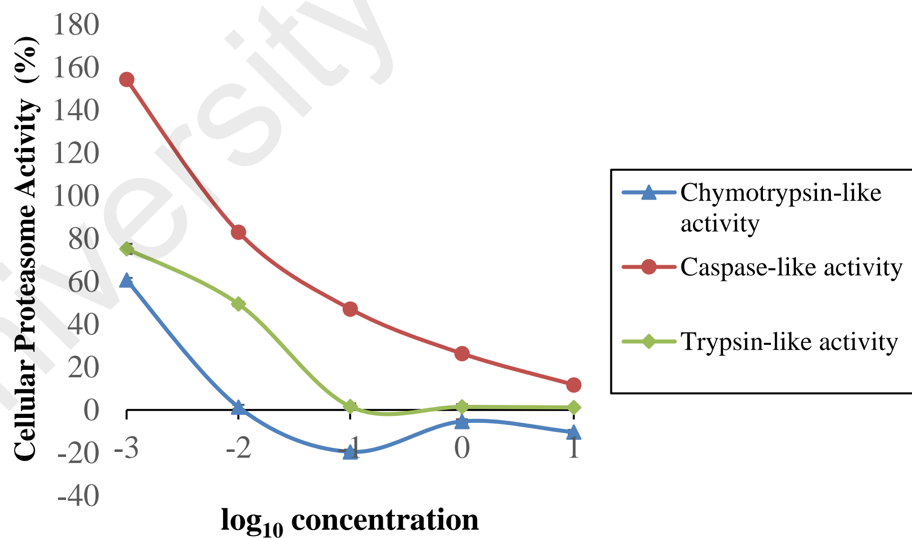


**Figure 4.22:** Comparison of three proteolytic activities of cellular proteasome in MDA-MB-231 cells after treatment with epoxomicin at different concentrations (0.001 – 10.0 µM).

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**Table 4.3:** $IC_{50}$ values of ACA and its analogues for cellular proteasomal activities.

| Proteolytic Activities | $IC_{50}$ (µM)* | | | |
|---|---|---|---|---|
| | ACA | AEA | AMCA | Epoxomicin |
| Chymotrypsin-like activity | 36.2± 5.3 | 32.8± 4.5 | 32.5± 4.9 | 0.002± 0.01 |
| Trypsin-like activity | 95.9± 6.4 | 111.3± 5.7 | 86.8± 3.9 | 0.0096± 0.008 |
| Caspase-like activity | N/A (64.4%) | N/A (53.6%) | N/A (68.4%) | 0.083± 0.02 |

* N/A denotes that $IC_{50}$ values were not attainable within the maximum tested concentration of said analogue, percentage in bracket (%) dictates percentage of proteasome activity upon maximum treatment with 200.0 µM of each compound.

### 4.3.3 Expression of Ubiquitinated Proteins

The major pathway of protein degradation in UPS uses ubiquitin as a marker that targets intracellular proteins for rapid proteolysis. Ubiquitin is a 76-amino-acid polypeptide that is highly conserved in all eukaryotes. Proteins are marked for degradation by the attachment of ubiquitin and the occurrence of the polyubiquitination process. The polyubiquitinated proteins are recognised and degraded by proteasome. Ubiquitin is released in the process to be reused in another cycle. If proteasome inhibition occurs, the ubiquitinated proteins cannot be degraded by proteasome and thus leads to accumulation of ubiquitinated proteins.

To explore the ubiquitination level of proteins, western blotting was conducted using specific antibody (PD41) against the ubiquitinated protein. Based on Figure 4.23, it was

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observed that the ubiquitination level of epoxomicin was highest, followed by AMCA and AEA. The expression of polyubiquitinated proteins was not so obvious upon treatment with ACA. This finding was consistent with the proteasomal inhibition potency as discussed in section 4.3.2. By referring to the inhibitory effects on proteasomal chymotrypsin-like activity in MDA-MB-231 cells, AMCA and AEA had lower $IC_{50}$ value than ACA, thus the ubiquitination level was increased with treatment of these two analogues. Collectively, AMCA and AEA were suggested to be involved in the proteasome inhibition and thus further block the protein degradation as evidenced by accumulation of ubiquitinated proteins.

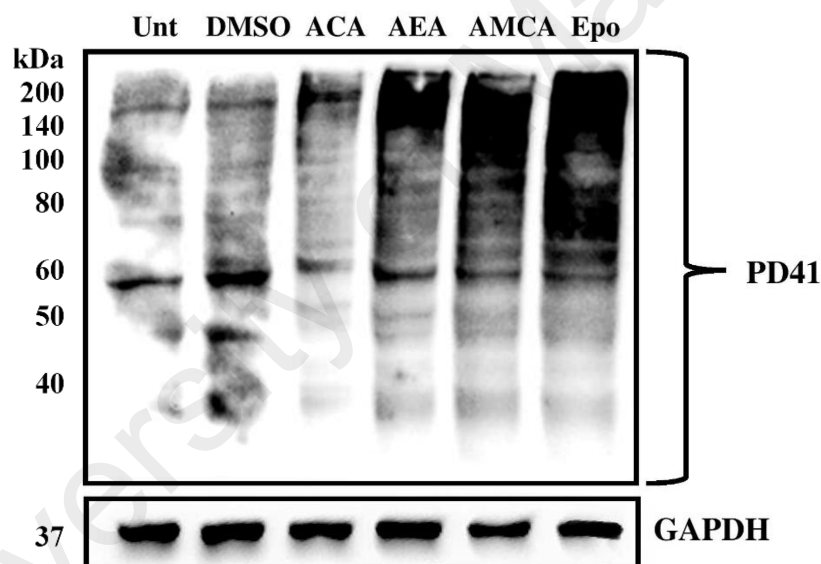


**Figure 4.23:** Western blotting analysis of ubiquitinated proteins in MDA-MB-231 cells upon treatment with ACA and its analogues. In first lane was untreated (Unt) protein, second lane was solvent control (DMSO) and last lane was positive control (Epo).

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## 4.4 *In silico* Docking

### 4.4.1 Docking of ACA Analogues to The Proteasomal β1 Subunit

To better understand the interaction between the structures of ACA and its analogues with the proteasome, *in silico* docking was performed to explore the computational interaction between ACA and its analogues with the three distinct β subunits of the proteasome. After docking ACA and its analogues to the β1, β2 and β5 active site using 100 genetic algorithms runs, AutoDock reports the best docking outputs (lowest docked free energy) for each GA run and also performs a cluster analysis in which the total number of clusters represents the reliability of docking results based on similarity of final docked conformation. The final docked energy was the sum of the internal energy of the ligand and the intermolecular energy. Different multiple clusters were analysed and a specific cluster rank was assigned. The criteria of choosing these ranks were based upon i) the distance from the carbonyl carbon to N terminal Thr must be within 4 Å; ii) the calculated docked free energy values that are favourable for binding of ACA and its analogues to the proteasome. A lower docking energy and a larger cluster would give a greater possibility prediction (Smith et al., 2004).

In docking studies of ACA to the proteasomal β1 subunit, 6 different multiple conformation clusters were analysed. Preference was given to multiple conformation clusters, whereby cluster rank 2 with 49 conformations was chosen. This was found consistent with the criteria of selection. As obtained from the AutoDock report, the final docked energy was found to be minimal in the selected cluster (−7.29 kcal/mol, Table 4.4). This places the carbon at 1'-acetoxy group of ACA to interact with the N terminal Thr1 at distance of 2.57 Å (Figure 4.24). For AEA, 6 multiple conformation clusters were analysed and the cluster rank 3 was selected with the lowest docked energy of −7.42 kcal/mol (Table 4.4). The carbonyl carbon attached to the 1'-acetoxy group of ACA was

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at the closest proximity of 2.23 Å to the N terminal Thr1, favouring the interaction with proteasome (Figure 4.25). In addition, AMCA was docked to β1 subunit and displayed 8 multiple conformation clusters. Out of the multiple conformation clusters, cluster rank 3 was selected for further analysis. The final docked energy to be minimal in this selected cluster was reported with the value of −7.51 kcal/mol (Table 4.4). Hence, this also places the carbon at 1'-acetoxy group at the shortest distance of 2.92 Å to N terminal of Thr1 (Figure 4.26).

Collectively, it was found that the 1'-aectoxy group of three analogues is very close the N-terminal of active site of the proteasome, and was believed to play an important role in the proteasomal inhibition.

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**Table 4.4:** *In silico* docking analysis of ACA analogues to the proteasomal β1 subunit.

| Compounds | No. of multiple-conformation clusters[a] | No. of conformations | Final energy intermolecular (kcal/mol) | Final internal energy (kcal/mol) | Torsional free energy (kcal/mol) | Free energy of binding (kcal/mol) | Final docked energy (kcal/mol)[b] | Distance between carbon and N terminal Thr1 (Å) |
|---|---|---|---|---|---|---|---|---|
| ACA | 6(2) | 49 | -6.67 | -0.62 | +1.79 | -4.88 | -7.29 | C13 (2.57) |
| AEA | 6(3) | 33 | -6.43 | -0.99 | +2.09 | -4.34 | -7.42 | C10 (2.23) |
| AMCA | 8(3) | 37 | -6.65 | -0.86 | +2.39 | -4.26 | -7.51 | C10 (2.92) |

[a] Number in bracket indicates the rank of cluster chosen.
[b] Final docking energy = Final intermolecular energy of the ligand + Final internal energy of the ligand.

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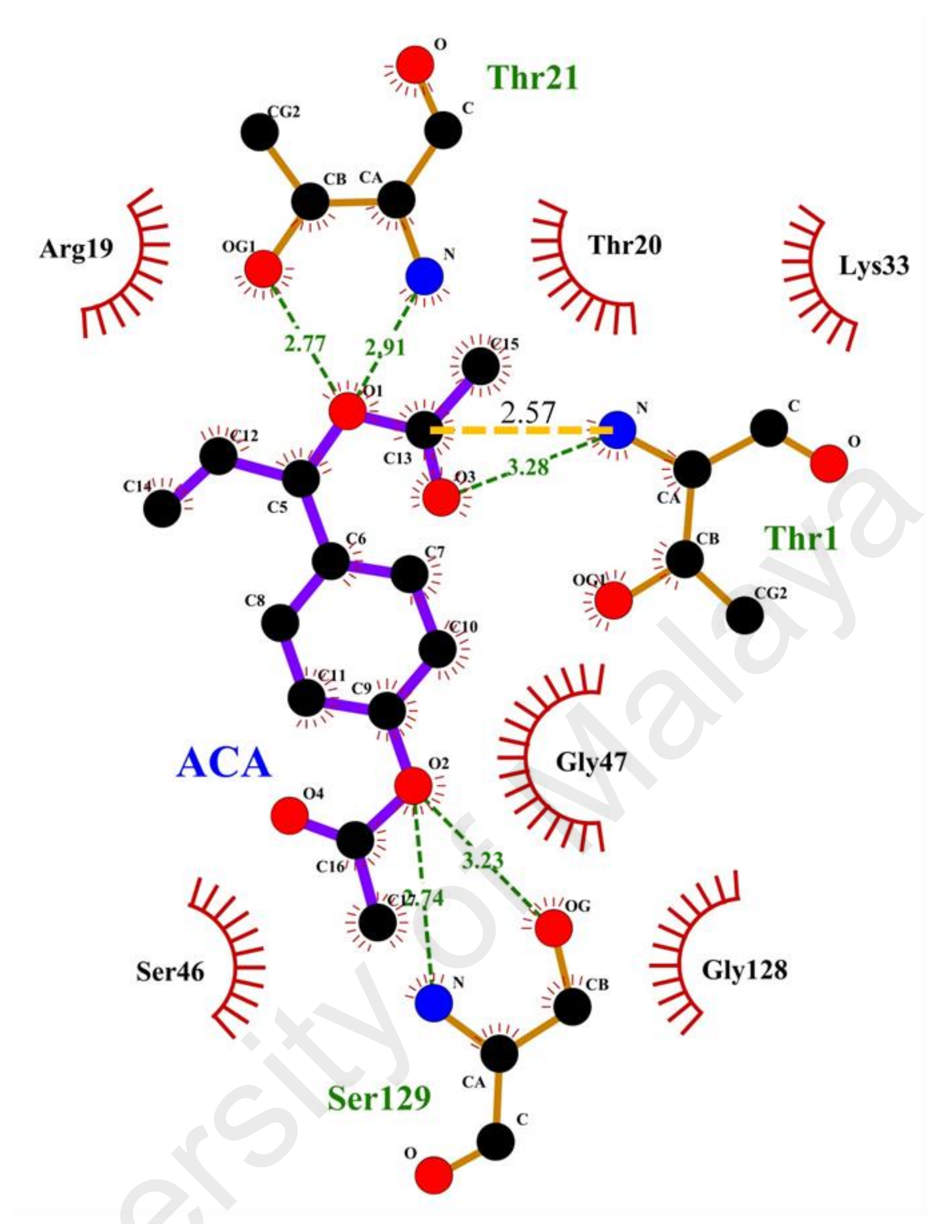


**Figure 4.24:** Ligplot analysis of proteasomal β1 subunit-ACA interaction.

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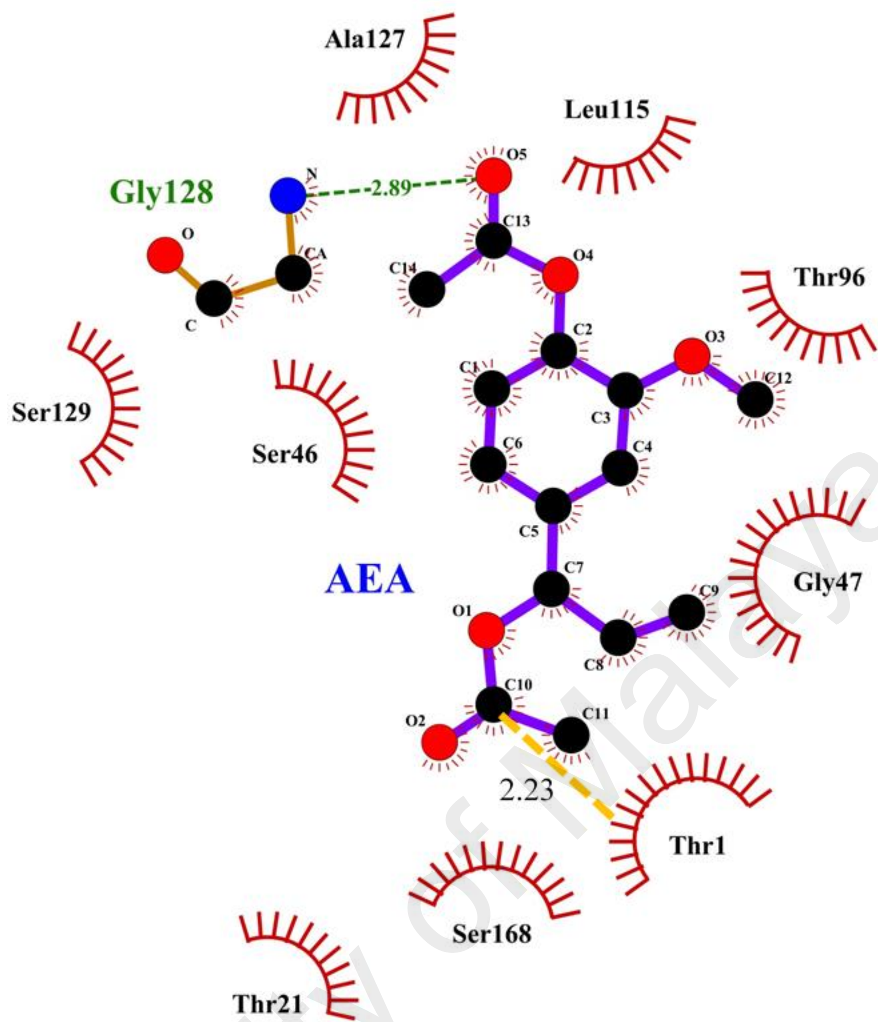


**Figure 4.25:** Ligplot analysis of proteasomal β1 subunit-AEA interaction.

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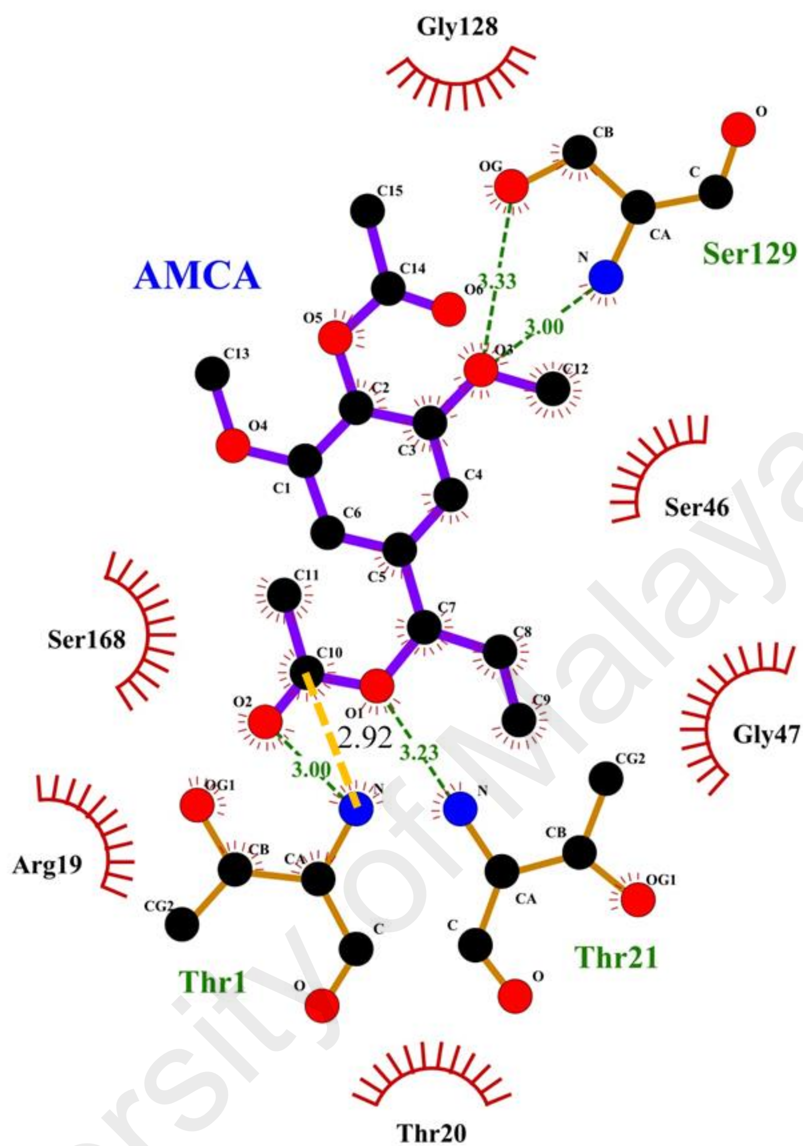


**Figure 4.26:** Ligplot analysis of proteasomal β1 subunit-AMCA interaction.

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### 4.4.2 Docking of ACA Analogues to The Proteasomal β2 Subunit

In order to understand the possible interaction between ACA, its analogues and the proteasome β2 subunit which is responsible for the trypsin-like activity, *in silico* docking analysis was carried out. The study revealed that ACA docked to β2 subunit with 3 multiple conformation clusters. Cluster rank 1 with 72 conformations was selected which had the lowest final docked energy in this cluster, that is, −8.08 kcal/mol (Table 4.5). The shortest distance between carbon in ACA and N terminal Thr1 of β2 subunit was 3.23 Å, which was found in the 4-acetoxy group (Figure 4.27). In the docking analysis of AEA to β2 subunit, 4 multiple conformation clusters were analysed and the cluster rank 2 was chosen. The data showed that the lowest docked energy was −8.75 kcal/mol (Table 4.5). The carbon at benzene ring which is attached with 4-acetoxy group was at the shortest distance with the N terminal Thr1 and has a value of 3.35 Å (Figure 4.28). Furthermore, 5 multiple conformation clusters were found in the docking analysis of AMCA with proteasomal β2 subunit. Cluster rank 1 was best suited and the minimum final docked energy was −9.18 kcal/mol (Table 4.5). The carbon bound to the 4-acetoxy group displayed the shortest proximity of 3.36 Å to N terminal of Thr1 (Figure 4.29).

In summary, the 4-acetoxy group is the key player that interacts with the β2 subunit of proteasome to inhibit the trypsin-like proteasomal activity.

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**Table 4.5:** *In silico* docking analysis of ACA analogues to the proteasomal β2 subunit.

| Compounds | No. of multiple-conformation clusters[a] | No. of conformations | Final energy intermolecular (kcal/mol) | Final internal energy (kcal/mol) | Torsional free energy (kcal/mol) | Free energy of binding (kcal/mol) | Final docked energy (kcal/mol)[b] | Distance between carbon and N terminal Thr1 (Å) |
|---|---|---|---|---|---|---|---|---|
| ACA | 3(1) | 72 | -7.37 | -0.71 | +1.79 | -5.58 | -8.08 | C16 (3.23) |
| AEA | 4(2) | 77 | -7.62 | -1.13 | +2.09 | -5.53 | -8.75 | C2 (3.35) |
| AMCA | 5(1) | 88 | -7.77 | -1.41 | +2.39 | -5.39 | -9.18 | C2 (3.36) |

[a] Number in bracket indicates the rank of cluster chosen.
[b] Final docking energy = Final intermolecular energy of the ligand + Final internal energy of the ligand.

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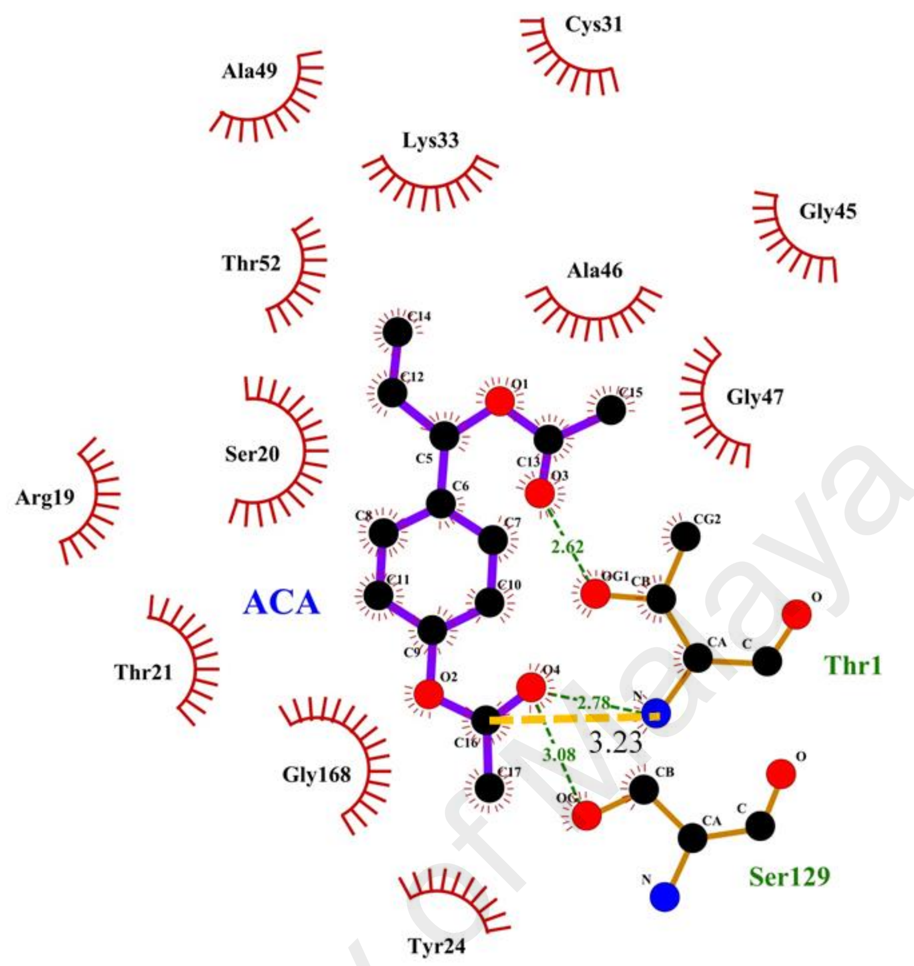


**Figure 4.27:** Ligplot analysis of proteasomal β2 subunit-ACA interaction.

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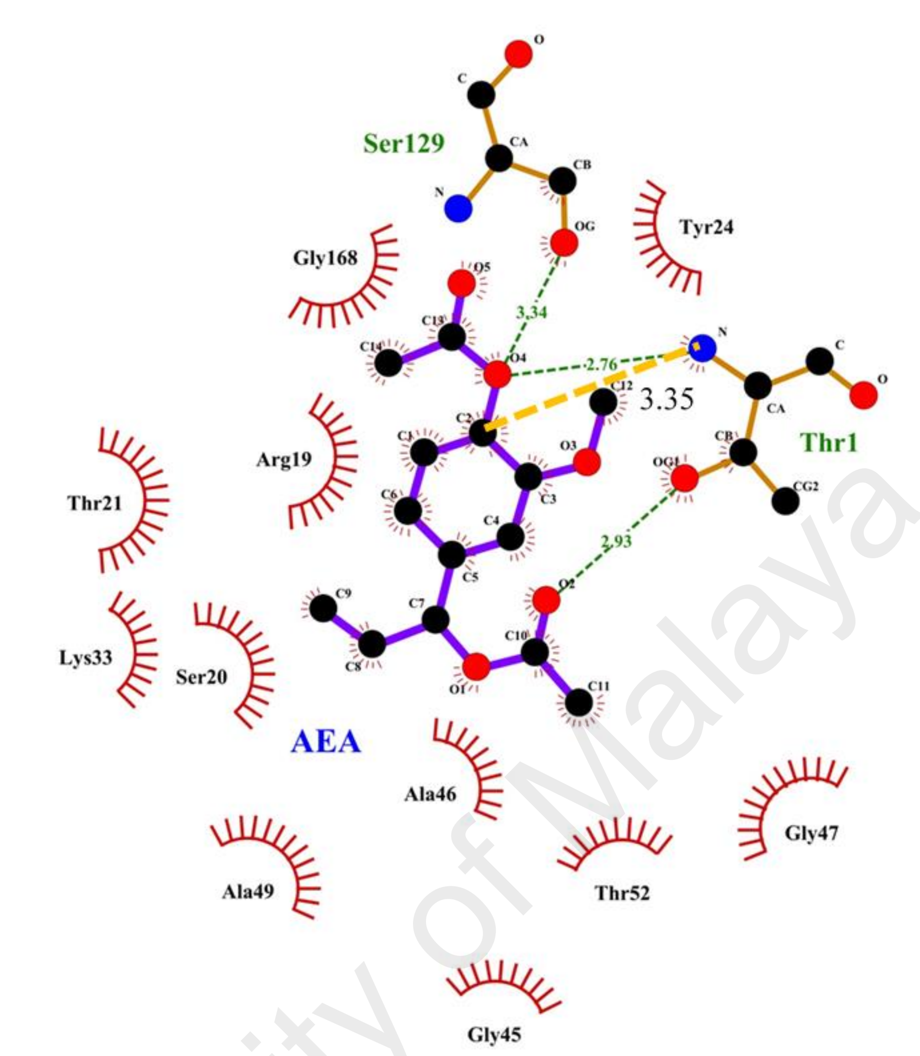


**Figure 4.28:** Ligplot analysis of proteasomal β2 subunit-AEA interaction.

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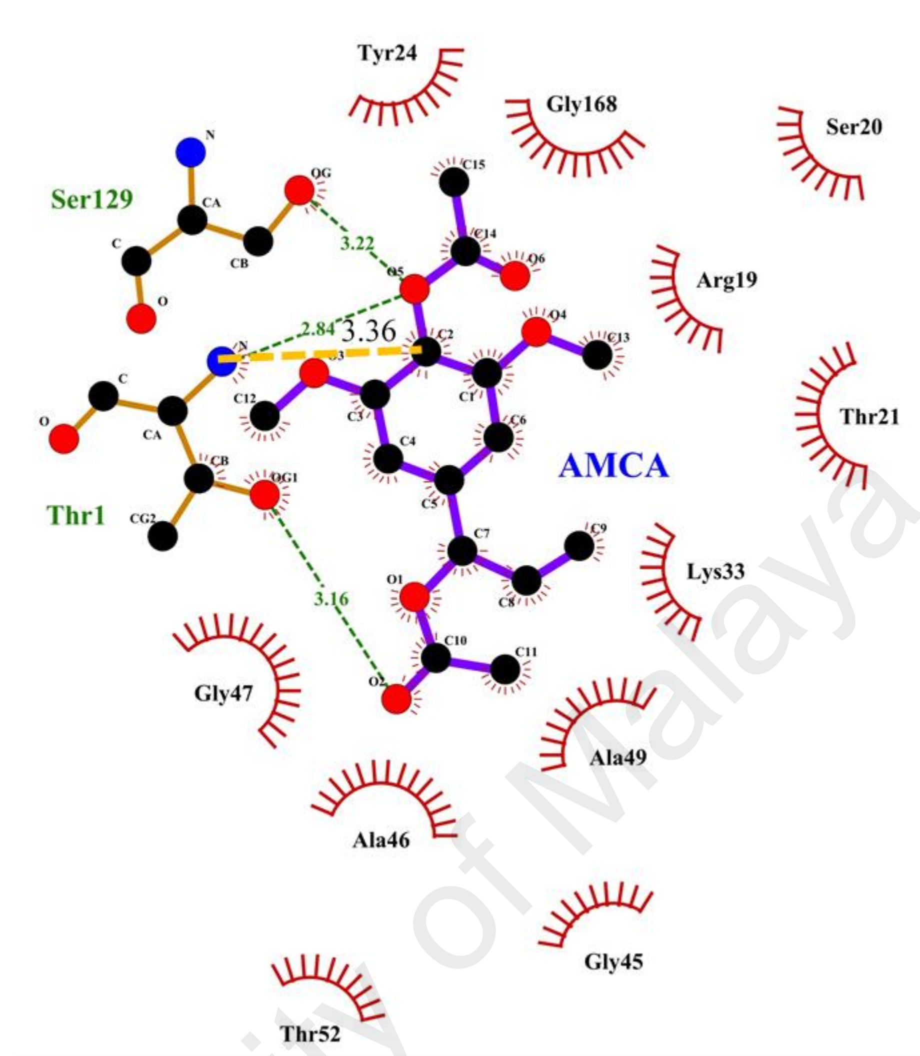


**Figure 4.29:** Ligplot analysis of proteasomal β2 subunit-AMCA interaction.

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### 4.4.3 Docking of ACA Analogues to The Proteasomal β5 Subunit

ACA and its analogues were able to inhibit the activity of purified 20S proteasome and cellular 26S proteasome. However, the involved molecular mechanism is unknown. For better understanding of the possible chemical nature of ACA and analogues to inhibit the chymotrypsin-like activity of the proteasome, each compound was docked to the active site of the proteasome β5 subunit, which is responsible for the chymotrypsin-like activity. In the docking analysis of ACA with proteasome β5 subunit, 5 multiple conformation clusters were analysed and cluster rank 4 was selected. The final docked energy was revealed to be minimal in this cluster (−6.97 kcal/mol, Table 4.6). This allows the carbon at 1'-acetoxy group of ACA dock with the N terminal Thr1 at distance of 2.48 Å (Figure 4.30). For AEA, 8 multiple conformation clusters were found and cluster rank 2 was selected for determination of lowest final docked energy. The energy used to dock AEA with β5 subunit was −7.95 kcal/mol (Table 4.6). The carbon at 1'-acetoxy group also showed the closest proximity of 2.82 Å to the N terminal Thr1 (Figure 4.31). Lastly, AMCA was docked to the β5 subunit with the outcome of 7 multiple conformation clusters. The cluster rank 1 was chosen and it showed the final docked energy to be the lowest in this cluster with the value of −8.85 kcal/mol (Table 4.6). The distance between carbon at 1'-acetoxy group with N terminal Thr1 was the shortest one, with the value of 2.70 Å (Figure 4.32).

In short, ACA, AEA and AMCA bind with the proteasomal β5 subunit, whereby the 1'-acetoxy group interact closely with the N terminal Thr1 of proteasomal β5 subunit.

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**Table 4.6:** *In silico* docking analysis of ACA analogues to the proteasomal β5 subunit.

| Compounds | No. of multiple-conformation clusters[a] | No. of conformations | Final energy intermolecular (kcal/mol) | Final internal energy (kcal/mol) | Torsional free energy (kcal/mol) | Free energy of binding (kcal/mol) | Final docked energy (kcal/mol)[b] | Distance between carbon and N terminal Thr1 (Å) |
|---|---|---|---|---|---|---|---|---|
| ACA | 5(4) | 34 | -6.23 | -0.74 | +1.79 | -4.45 | -6.97 | C13 (2.48) |
| AEA | 8(2) | 37 | -7.04 | -0.91 | +2.09 | -4.95 | -7.95 | C10 (2.82) |
| AMCA | 7(1) | 39 | -7.52 | -1.33 | +2.39 | -5.14 | -8.85 | C10 (2.70) |

[a] Number in bracket indicates the rank of cluster chosen.
[b] Final docking energy = Final intermolecular energy of the ligand + Final internal energy of the ligand.

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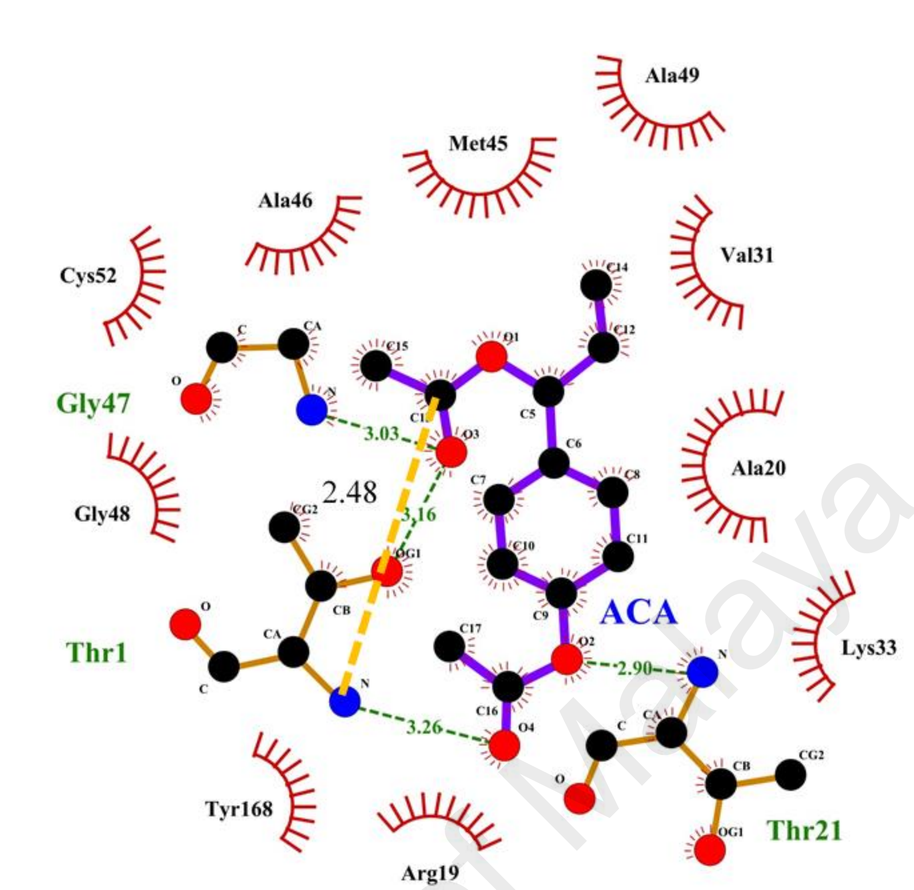


**Figure 4.30:** Ligplot analysis of proteasomal β5 subunit-ACA interaction.

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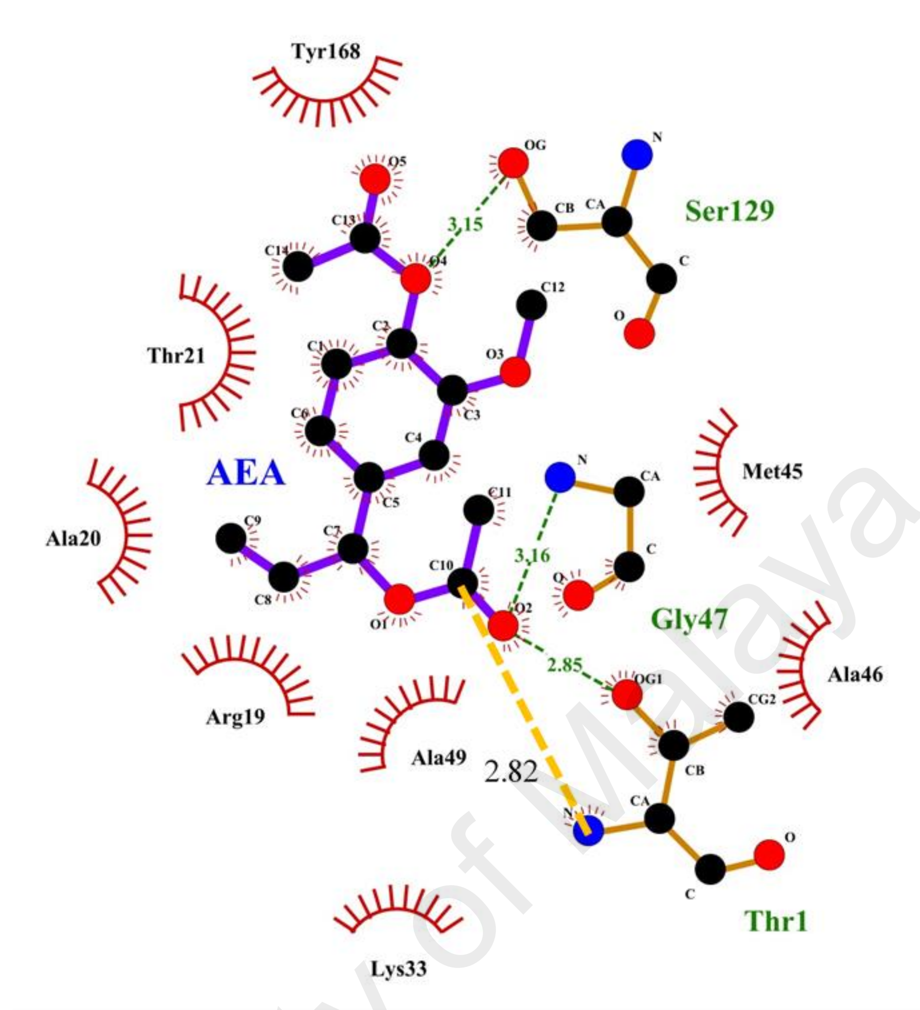


**Figure 4.31:** Ligplot analysis of proteasomal β5 subunit-AEA interaction.

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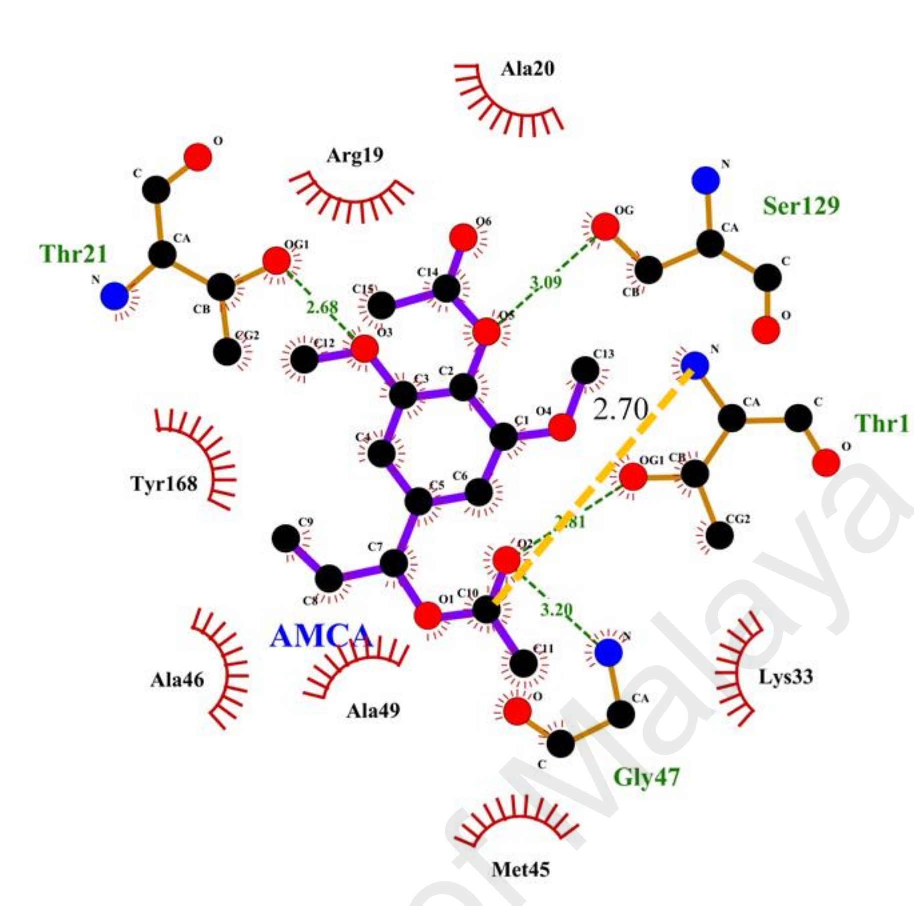


**Figure 4.32:** Ligplot analysis of proteasomal β5 subunit-AMCA interaction.

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### 4.5 Determination of Apoptosis

#### 4.5.1 DNA Fragmentation Assay

To determine whether ACA and its analogues could potentiate apoptosis-mediated cell death in MDA-MB-231 cells, the DNA fragmentation assay performed.

Figure 4.33 demonstrated DNA from the untreated group showed no degradation, whereas DNA laddering was observed in MDA-MB-231 cells that were treated with ACA, AEA and AMCA at their $IC_{50}$ concentrations. This typical oligonucleosomal DNA degradation is one of the hallmarks of apoptotic cell death, thus it was suggested that ACA, AEA and AMCA induce cell death through apoptosis.

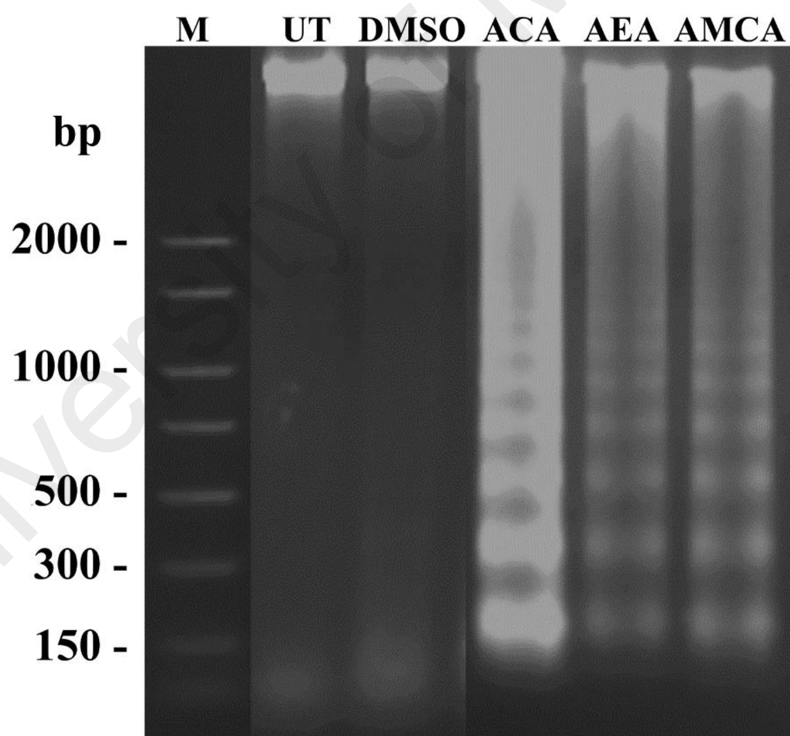


**Figure 4.33:** Confirmation of apoptosis-mediated cell death using the DNA fragmentation assay. MDA-MB-231 cancer cell line was treated with ACA and its analogues for 24 hrs and isolated DNA was observed on 1.0% (w/v) agarose gel electrophoresis. M: DNA marker; UT: untreated lane; DMSO: solvent control.

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### 4.5.2 PARP Cleavage Assay

Further confirmation on the apoptosis-inducing effects of ACA, AEA and AMCA on cancer cells was conducted using the PARP assay, which measures the enzymatic cleavage of PARP following caspase activation. PARP can be cleaved by many caspases and is one of the main cleavage targets of caspase-3 (Cohen, 1997). Evaluation of PARP cleavage levels illustrates cellular disassembly and generally serves as a marker of cancer cells undergoing apoptosis (Oliver et al., 1998).

As shown in Figure 4.34, the cleavage of full length PARP enzymes (116-kDa) into a large (89-kDa) subunit protein was observed in the MDA-MB-231 cells that had been treated with ACA, AEA and AMCA at their $IC_{50}$ concentrations. Based on densitometry analysis, these three compounds increased the expression level of cleaved PARP by 7.8-, 4.8- and 1.8-fold compared to untreated cells, respectively (Figure 4.35).

These western blotting results confirmed that occurrence of apoptosis-mediated cell death via caspase-3 dependent activation induced by ACA, AEA and AMCA on human breast cancer cells *in vitro*. This was found to be consistent with previous DNA fragmentation apoptosis-confirmation assay.

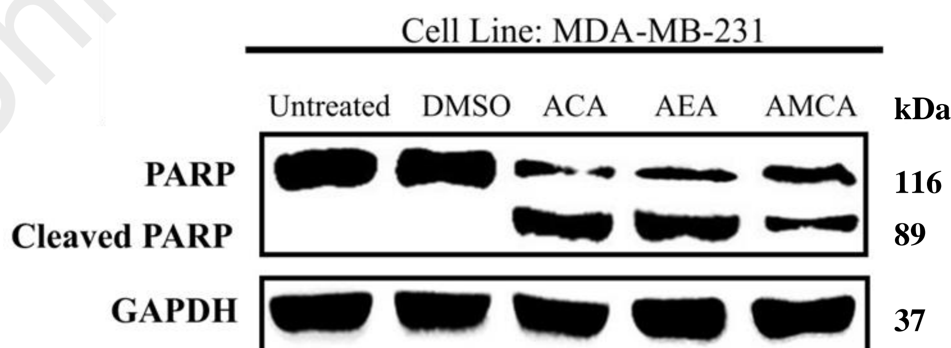


**Figure 4.34:** Indication of apoptosis-mediated cell death through the activation of caspase-3 leading to cleavage of full length PARP enzymes (116-kDa) into a large (89-kDa) subunit protein. GAPDH (37-kDa) was used as a normalisation control to ensure equal protein concentrations across samples. DMSO: solvent control.

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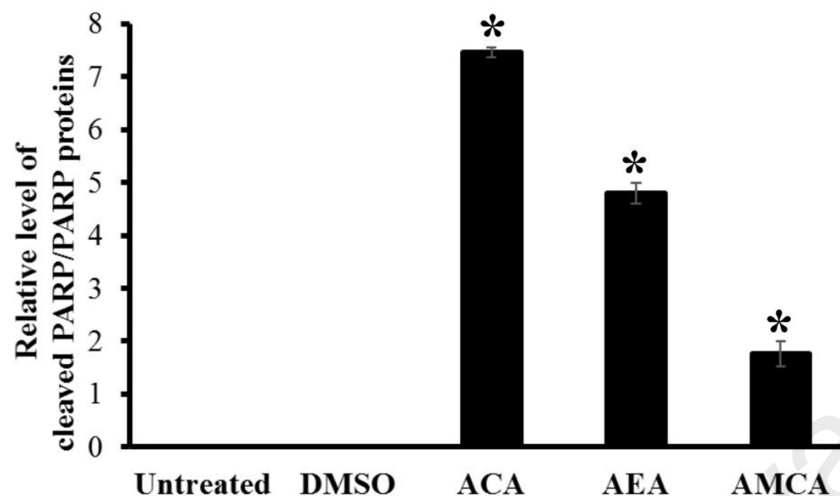


**Figure 4.35:** Densitometry analysis of the western blots for cleaved PARP/PARP proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting *p*-values ≤ 0.05.

### 4.5.3 Expression of Apoptosis-Related Proteins

Western blot analysis of MDA-MB-231 cells treated with ACA analogues was carried out to observe the effects on apoptosis-related proteins. ACA, AEA and AMCA were found to decrease Bcl-2 and Bcl-xL anti-apoptotic protein levels, and increase the expression level of p53 and pro-apoptotic Bax (Figure 4.36). As shown in Figures 4.37 and 4.38, treatment of ACA, AEA and AMCA at their $IC_{50}$ concentrations for 24 hrs significantly decreased the expression level of Bcl-2 by approximately 40-50% and Bcl-xL by around 30-50% when compared to untreated cells. The p53 levels in MDA-MB-231 cells increased by 3.3-, 3.4- and 2.3-fold after treatment with ACA, AEA and AMCA, respectively (Figure 4.39). The three compounds upregulated the expressions of pro-apoptotic Bax by 6.2-, 9.4-, 2.9-fold, respectively (Figure 4.40). Collectively, this suggested that PARP, p53, Bcl-2, Bcl-xL and Bax were involved in inducing apoptosis via the intrinsic pathway when treated with ACA, AEA and AMCA.

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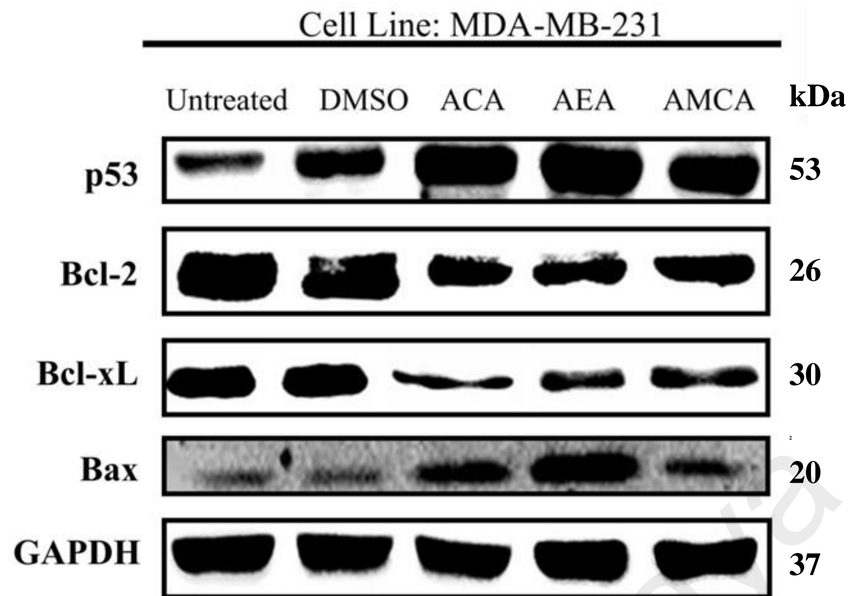


**Figure 4.36:** Western blotting analysis of apoptosis-related proteins in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. DMSO: solvent control; GAPDH housekeeping protein used for normalisation of protein levels.

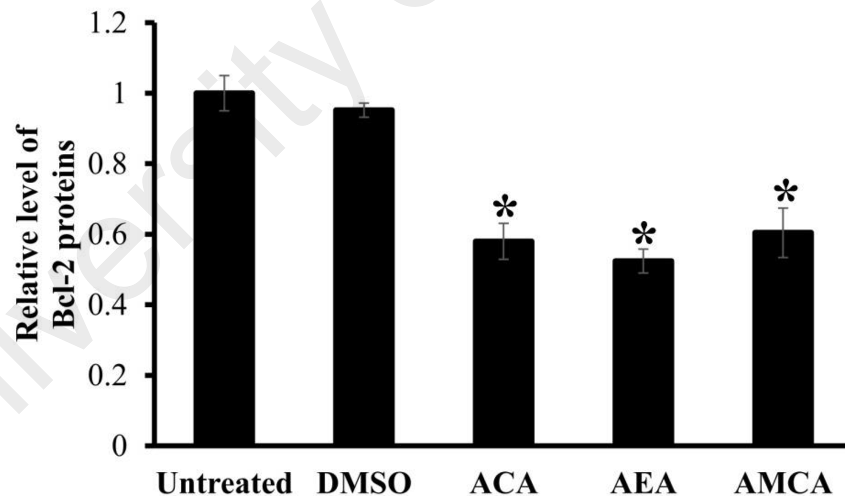


**Figure 4.37:** Densitometry analysis of the western blots for Bcl-2 proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting *p*-values $\leq 0.05$.

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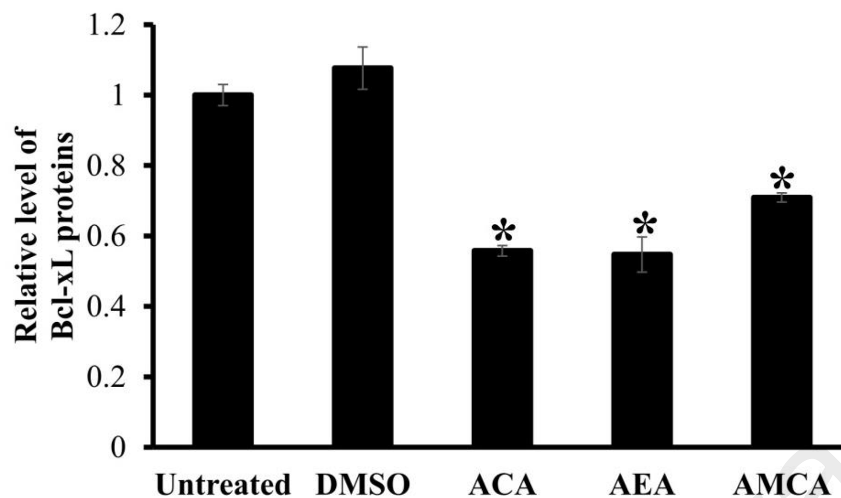


**Figure 4.38:** Densitometry analysis of the western blots for Bcl-xL proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting *p*-values ≤ 0.05.

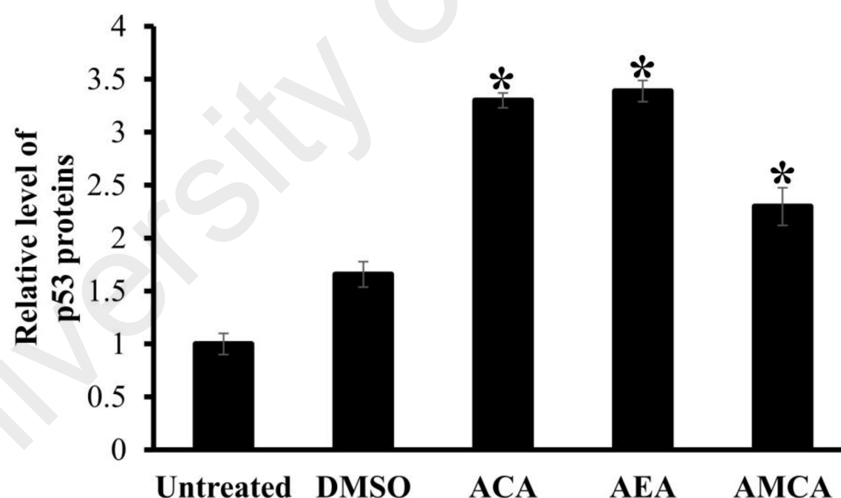


**Figure 4.39:** Densitometry analysis of the western blots for p53 proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting *p*-values ≤ 0.05.

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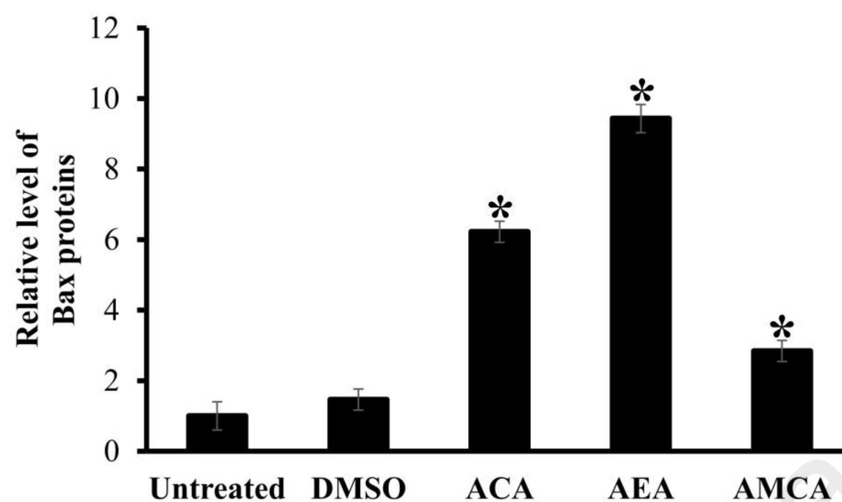


**Figure 4.40:** Densitometry analysis of the western blots for Bax proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting *p*-values ≤ 0.05.

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## 4.6 Migration

### 4.6.1 Wound healing

Cell migration is an important step in the metastatic process (Harlozinska, 2005). Therefore, the effects of ACA and its analogues on cell migration were also examined on breast cancer MDA-MB-231 cells. This cell line was chosen for anti-migration study due to its highly aggressive and strongly metastatic properties among the selected panel of cell lines (Price et al., 1990; Nair et al., 2004).

According to quantification on open wound areas after 24 hrs of incubation using the TScratch software in Figure 4.41, compound **7** was found to reduce MDA-MB-231 cell migration rates the most whereby the area of scratch wounds healed by 13.0 ± 1.0% compared to 29.0 ± 3.0% in untreated controls. Meanwhile, ACA and its cytotoxic analogues, AEA and AMCA were found to reduce MDA-MB-231 cell migration rates at 18.0 ± 4.0%, 20.0 ± 3.0% and 17.0 ± 2.0%, respectively. The data showed that ACA,

AEA, AMCA, compounds **5-8** and **20-21** significantly inhibited the migration rate of MDA-MB-231 cells compared with untreated cells (Figure 4.42).

Furthermore, the result revealed that the difference in inhibition of cell migration between untreated cells and cells treated with DMSO was insignificant ($p$-value > 0.05) (Appendix F), suggesting that anti-migratory effects of tested compounds were not due to the solvent.

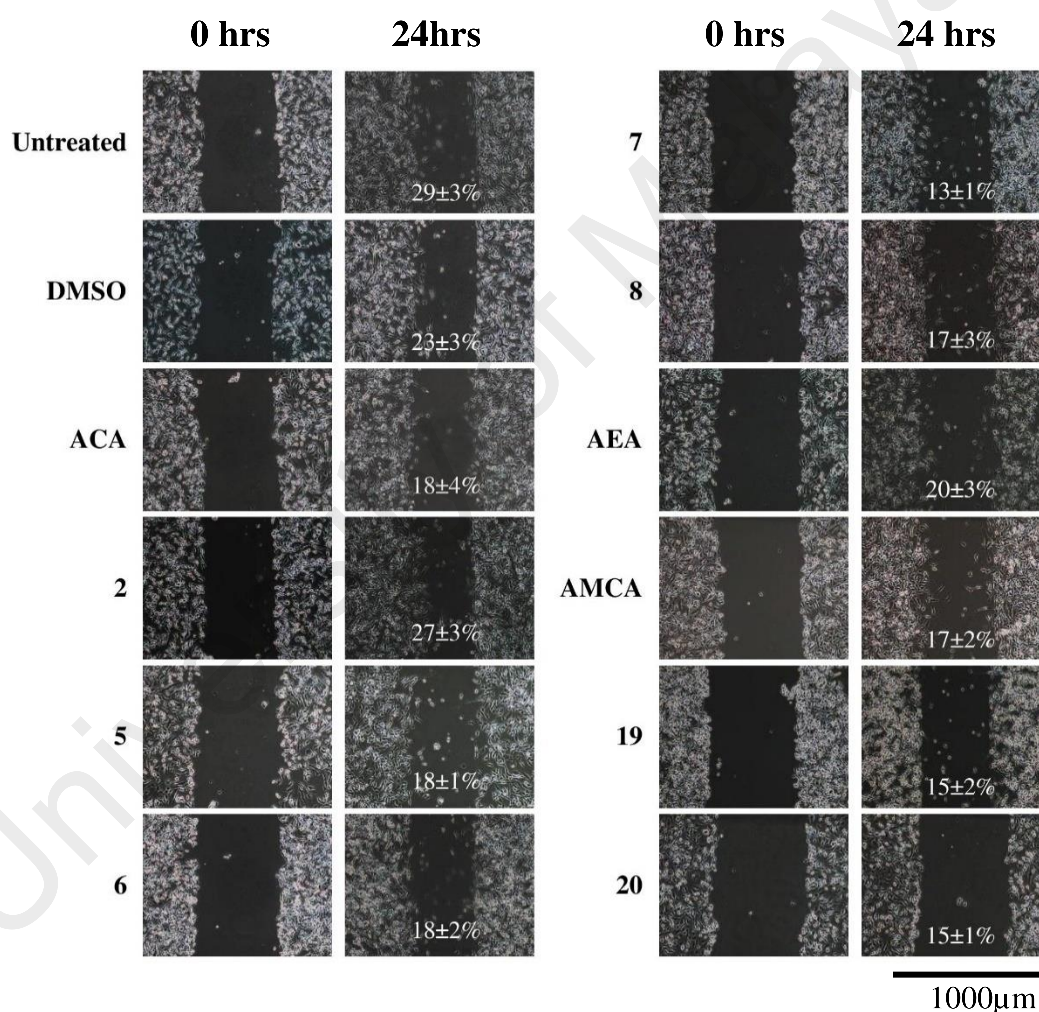


**Figure 4.41:** Representative photos of wound-healing assay of MDA-MB-231 cells after various treatments of ACA and its analogues at $IC_{20}$. The open wound area at 24 hrs was quantified using TScratch software relative to wound area at 0 hrs. All data are presented as mean ± SEM from three independent replicates, with representative images at 40× magnification shown from each treatment group. Scale bar apply for all pictures. DMSO: solvent control.

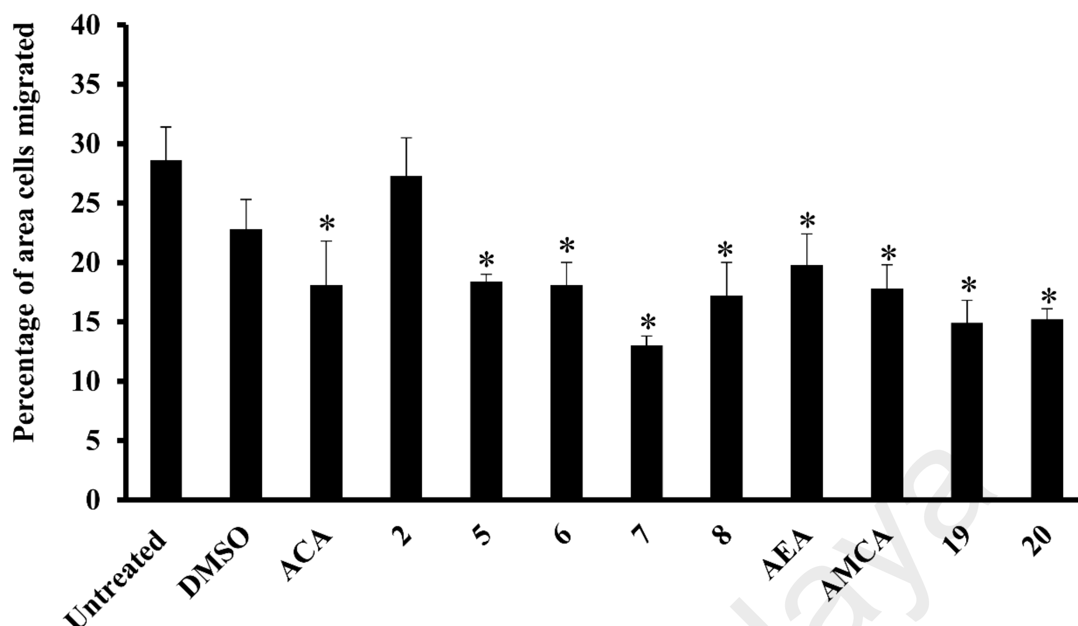


**Figure 4.42:** Effects of ACA and its analogues on the cell migration of MDA-MB-231 cells. The open wound area at 24 hrs was quantified using TScratch software relative to wound area at 0 hrs. The mean percentage of area migrated ± SEM from three separate experiments are illustrated. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting $p$-values ≤ 0.05. DMSO: solvent control.

### 4.6.2 SAR Analysis on Anti-Migration Activity

Comparison of the association between the biological activity of ACA and its analogues via SAR studies towards their anti-migration potency was also conducted. The SAR study showed that the contribution of acetoxy group at 1'-position is important but not compulsory for anti-migration activity. Apparently, analogue **2** which was devoid of 1'-acetoxy group did not show significant results in the inhibition of cell migration. However, significant anti-migrations were also displayed upon treatment of some analogues (**5**, **6** and **8**) which lacked the acetoxy group at 1'-position. The next structural feature considered was the substituents at 3-position. Five analogues (**6**, **8**, AEA, AMCA and **19**) with 3-methoxy group inhibited migration of cancer cells significantly. Nevertheless, analogue **2** with 3-methoxy group did not inhibit cell migration. This

depicted the existence of 3-methoxy group as an optional feature for cell migration. SAR studies also suggested that the presence of a hydroxy group at 4-position distinctively reduced or abolished the anti-migration activity. This discovery was in accordance with data shown in Figure 4.42, where analogue **2** with 4-hydroxy group eliminated anti-migration effects on all cancer cells tested. All of the compounds (ACA, AEA, AMCA, **6** and **8**) with 4-acetoxy group showed significant anti-migration effects on the cancer cells. Thus, acetoxy group at 4-position is deemed compulsory for anti-migration activity. Analogue **2** with a 5-methoxy group were found to be lacking in anti-migration activity. Based on Figure 4.43, the structural factors of ACA and its analogues governing cancer cell migration properties are concluded as follows: (1) The 1'- and 4-acetoxy groups are essential structural components. (2) The presence of the 3-methoxy group increases the activity. (3) The substitution of 4-acetoxy group with 4-hydroxy group and presence of 5-methoxy group reduces the activity.

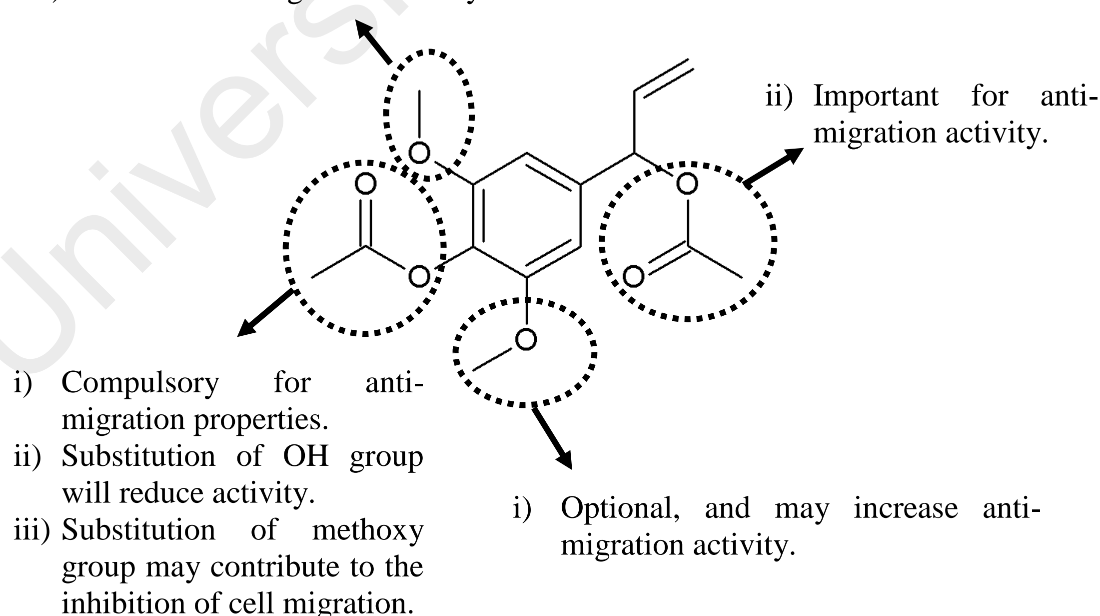


**Figure 4.43:** SAR analysis of individual chemical structure groups within ACA and its analogues towards anti-migration properties on MDA-MB-231 cell line.

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### 4.6.3 Expression of Metastasis-Related Proteins

In addition to anti-proliferative effects and induction of apoptosis, ACA, AEA and AMCA also showed significant anti-migratory effects on MDA-MB-231 breast cancer cells. Thus, the molecular mechanisms related to the anti-migratory effects of these compounds were also investigated. The effect of these compounds on the expression of integrin β1, which is a major player in tumour metastasis that mediate the adhesion of cells on extracellular matrix (ECM) (Jin & Varner, 2004) and some of its downstream molecules were determined. The integrin-mediated pathway on MDA-MB-231 breast cancer cells was determined by western blot analysis and it was found that the level of integrin β1 decreased following ACA, AEA and AMCA treatment (Figure 4.44). Upon integrin β1 binding to ECM, the downstream signalling molecules such as, FAK and Akt are phosphorylated (Vachon, 2011) and participated in the regulation of cell migration. In analysing the integrin-induced signalling pathway, phosphorylation of FAK and Akt was significantly reduced by ACA, AEA and AMCA after 24 hrs (Figure 4.44). As shown in Figure 4.45, the integrin β1 level of MDA-MB-231 cells decreased by 15.5, 37.1 and 93.3% after treatment with ACA, AEA and AMCA, respectively. The reduction level of phosphorylated FAK and Akt by ACA, AEA and AMCA was about 70-94% (Figures 4.46 and 4.47).

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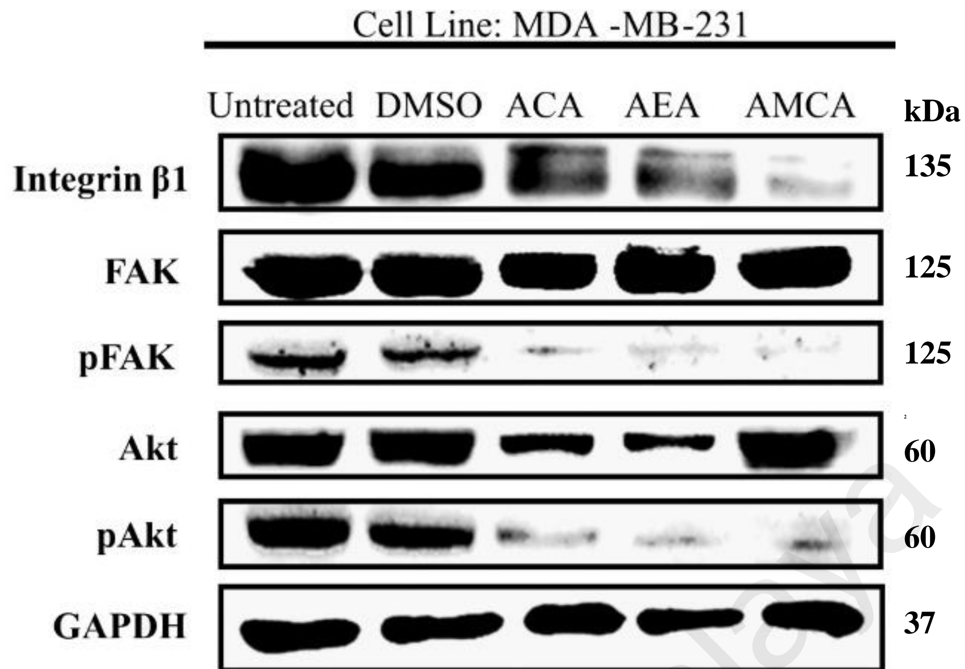


**Figure 4.44:** Western blotting analysis of metastasis-related proteins in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. DMSO: solvent control; GAPDH housekeeping protein used for normalisation of protein levels.

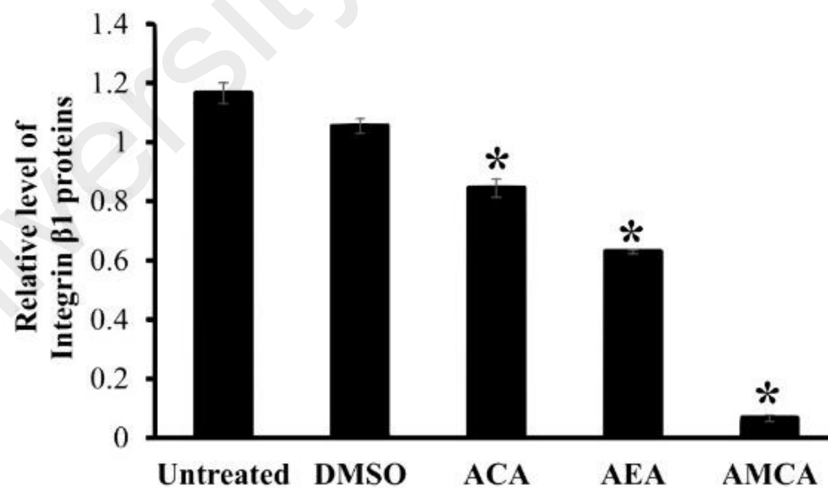


**Figure 4.45:** Densitometry analysis of the western blots for integrin β1 proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting $p$-values ≤ 0.05.

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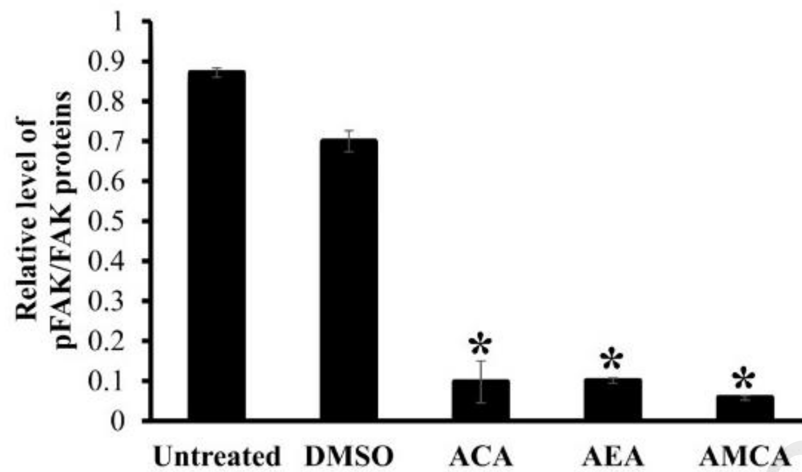


**Figure 4.46:** Densitometry analysis of the western blots for pFAK/FAK proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting $p$-values ≤ 0.05.

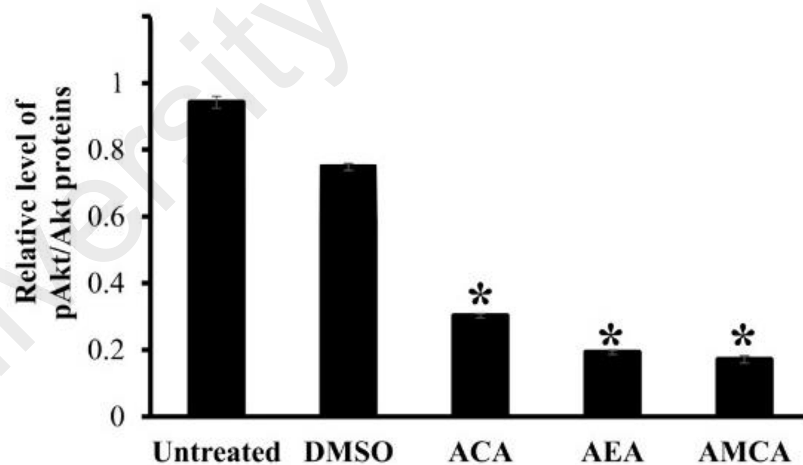


**Figure 4.47:** Densitometry analysis of the western blots for pAkt/Akt proteins expression in MDA-MB-231 cells treated with ACA and its analogues for 24 hrs. Statistically significant differences were compared between untreated conditions versus treatment groups with (*) denoting $p$-values ≤ 0.05.

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# CHAPTER 5: DISCUSSION

Cancer is rapidly becoming a global pandemic, with more than 14 million people affected worldwide. Despite the advances in the field of cancer research, many countries are experiencing the unpleasant conditions of this deadly disease, and still there is a continuous need to discover and develop more efficient and cost effective anti-cancer agents. The plant based natural products have been recognised to possess the richest source of high chemical diversity to initiate the drug design regime for identification of novel structures. Thus, researchers concentrated efforts to discover and develop natural products as the promising cancer therapeutic agents. However, some obstructions including low quantity of extracted natural products, insufficient natural resources and high complexity of the compound structure restrain the production of natural products for the usage in cancer therapy. Thus, from this point of view, hemi-synthesis of new anti-cancer agents that are highly effective, low cost and have minor environmental impact would be the best strategy in cancer management and treatment. In addition, the modified compounds with distinct molecular mechanisms are considered more favourable for higher efficacy and better survival. The rationale for hemi-synthesis of compounds is to relate the effects of various substituents on different biological activities of the compound. As a consequent, there is an increase in the number of studies involving SAR analysis on anti-cancer effects with chemically modified compounds. The SAR data could serve as the basis for development of better anti-cancer agents to improve cancer treatment outcome.

Previously, the use of the natural product, ACA, was shown to exert anti-cancer effects by inducing apoptosis (Ito et al., 2004) and cell cycle arrest (Awang et al., 2010) or inhibiting angiogenesis (Pang et al., 2011) and metastasis (Wang et al., 2014). Moreover, previous studies also showed that ACA and its natural analogue, AEA induce the extrinsic

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apoptosis-mediated cell death pathway in cancer cells via dysregulation of NF-κB (Awang et al., 2010; Hasima et al., 2010; In et al., 2011). The blockade of NF-κB has been revealed to induce apoptosis of cancer cells and was suggested as a potential target in cancer treatment (Hoffmann et al., 2002). Under certain conditions during tumourigenesis, the inhibitor IκB proteins are degraded, which then allow the activation of NF-κB (Karin & Ben-Neriah, 2000). This degradation is mediated by the ubiquitin-proteasome system, which can be blocked by a proteasome inhibitor (Kisselev & Goldberg, 2001). Taken from the ability of proteasome inhibitor to block NF-κB activation, it has been suggested to induce apoptosis on cancer cells (Mayo & Baldwin, 2000). Although previous studies discovered that ACA induces apoptosis and inhibits migration on some cancer cells, the apoptotic induction via UPS and anti-migration mechanisms of hemi-synthetic ACA analogues on cancer cells have never been reported.

In the present study, it was found that not only ACA, but two of its analogues, AEA and AMCA were able to induce apoptosis and suppress the migration of MDA-MB-231 breast cancer cells. However, these compounds did not exert effective anti-cancer effects via regulation of UPS. Nevertheless, deduction of important functional groups governing the anti-proliferation, proteasome inhibition and anti-migration effects provides an insight into the potential interactions involved between a compound and cancer targets. In conclusion, acetoxy group either attached at 1'- or 4- position of the benzene ring play an important role in anti-proliferation, proteasome inhibition and anti-migration effects. Another substituent, methoxy group attached at 3- position of benzene ring also contributes to the anti-proliferative and anti-migration activities.

The molecular mechanisms of the effects of ACA analogues on apoptotic induction and anti-migration activities were elucidated. The western blot analysis showed that expression levels of cleaved PARP, p53 and Bax increased, while the expression levels

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of Bcl-2 and Bcl-xL reduced after treatment, suggesting that ACA and its analogues-induced apoptosis was regulated via the mitochondrial pathway. In addition, ACA, AEA and AMCA decreased the expression levels of pFAK/FAK and pAkt/Akt using the integrin ß1-mediated signalling pathway, ultimately leading to inhibition of cell migration.

In brief, the natural compound ACA and its hemi-synthetic analogues, AEA and AMCA are promising agents for cancer therapy applications, especially for inhibition of cancer cell growth, apoptotic induction and suppression of migration.

## 5.1 ACA and Its Analogues in Relation to Anti-Proliferative Activities

In this study, MTT assays were conducted with two objectives, firstly, in determining which of the ACA analogues were able to demonstrate significant cytotoxic activity, and secondly, to determine the $IC_{50}$ values of each active compounds, which is crucial in subsequent downstream assays.

The MTT assays showed that only ACA, AEA and AMCA induced anti-proliferative effects on MDA-MB-231 breast cancer cells, whereas ACA and AEA also inhibited the growth of MCF-7, RT-112, EJ-28, PC-3, HSC-4 and HepG2 cancer cell lines with no cytotoxic effects on HMEC normal human breast cells. Due to the differential activity of ACA and AEA, the selectivity index (SI) was calculated to indicate their degree of selectivity (SI = $IC_{50}$ normal cell / $IC_{50}$ cancer cell). The greater the SI value is, the more selective it is. Since $IC_{50}$ of the compounds on HMEC normal human breast cells was more than 50.0 µM, thus the SI values of ACA and AEA were higher than 2. This suggested that these compounds could kill the cancer cells with lower drug doses and exert fewer side effects on non-cancerous tissue.



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Although data from MTT assays clearly showed that cancer cells were dying following exposure to these compounds, it was also noted that the rate of killing between cell lines varied, as observed with the different $IC_{50}$ values obtained. It is proposed that these variations are brought upon by differences in the alternative genetic paths these cancer cell lines take to turn malignant. For example, the expression level of certain genes involved in drug retention (*i.e.* drug efflux and influx) within the cell would modulate the exposure time for each cancer cell line to react towards the drug in question. The intracellular balance between tumour suppressor genes pushing towards apoptosis against oncogenes leaning towards anti-apoptosis and proliferation was also likely to play a role in creating a diversified microenvironment influencing the outcome on how each cancer cell line reacts towards an anti-cancer agent. Moreover, the aggressiveness of each cancer genotype would also without doubt, regulate the minimal dosage of ACA and its analogues required to achieve $IC_{50}$ levels in respective cancer types.

MDA-MB-231 was reported as the most sensitive cell line towards the compounds tested in terms of cytotoxicity, and was thus selected as a model for further investigation. Another type of breast cancer cell, MCF-7 cell line was only sensitive to ACA and AEA. Despite both cell lines being breast cancer cell lines, they are different in genotypes, MDA-MB-231 is a triple negative, lack of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) expression breast cancer cell line, while MCF-7 is an ER and PR positive breast cancer cell line. Thus, these differences could be the underlying cause towards response variations and drug sensitivity (Rouzier et al., 2005).

## 5.2 ACA and Its Analogues in Relation to Ubiquitin-Proteasome System

Over a decade, proteasome inhibition has emerged as an effective strategy in the treatment of cancer. It has been revealed that proteasome inhibition is related with

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induction of apoptosis in cancer cells, but not in normal cells (Dou & Li, 1999). Proteasomes are important for cell protection from apoptosis by maintaining the balance of anti-apoptotic and pro-apoptotic proteins (Kloetzel, 2001; Ling et al., 2002). Therefore, the interest in potent and specific proteasome inhibitors that may be used as potential anti-cancer drug is very high.

ACA and its analogues, AEA and AMCA were tested for their inhibitory capacity in purified 20S proteasome and cellular proteasome of MDA-MB-231 cells. The specific inhibition of a single catalytic site is of special interest for drug development, therefore, the inhibition of the three different proteasomal activities were analysed. The specific substrates used for the different catalytic activities are Suc-LLVY-Glo™ Substrate for chymotrypsin-like activity, Z-LRR-Glo™ Substrate for trypsin-like activity and Z-nLPnLD-Glo™ Substrate for caspase-like activity.

ACA and AEA inhibited 20S proteasomal chymotrypsin-like activities with $IC_{50}$ values less than 200.0 μM. This indicated that proteasomal inhibition by ACA and AEA only can be achieved with higher doses of compound. Notably, epoxomicin inhibited all three proteasomal activities with $IC_{50}$ values below 3.5 μM. The huge difference in the proteasomal inhibition between ACA, its analogues and the commercial proteasome inhibitor could be due to the binding ability and the impact on 20S proteasome. On the other hand, compared to 20S proteasome, ACA and its analogues exerted more effective inhibition on cellular proteasome in MDA-MB-231 cells. It was suggested that it may be due the stability of ACA analogues in cells and/ or existence of other ACA-binding proteins. Unfortunately, the proteasomal inhibition effects of ACA and its analogues are significantly weaker than epoxomicin.

Cancer cells are often more sensitive to proteasomal inhibitors than normal cells. The clinically approved proteasomal inhibitor bortezomib suppressed growth and induced

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apoptosis in the sensitive cancer cells, whereas the normal cells tolerate higher inhibitor concentrations (Hideshima et al., 2001). However, the differences in cellular properties and resistance mechanisms with bortezomib require further development of new proteasomal inhibitors. Despite the modification of structures made on ACA, the proteasomal inhibition was not as effective as the commercial proteasome inhibitor, epoxomicin and this could be contributed by the permeation ability of the compounds.

Molecular docking was widely used in the discovery and development of proteasome inhibitors. This methodology provides better understanding for the proteasome-ligand interactions. Protein-ligand molecular docking is a computational method for predicting the best position of how a ligand binds in a protein binding pocket (Liao et al., 2011). Protein-ligand docking mimics the recognition process in which a small molecule (ligand) translates, rotates and twists thoroughly in the active site of a macromolecule (protein) with the aim of finding the energetically most suitable conformation binding mode (search algorithm), being the protein-ligand affinity (binding energy) estimated by a scoring function (scoring algorithm) (Gallastegui et al., 2012; Zhu & Li, 2012).

In 2003, Kazi and friends performed computational docking studies using Autodock software and the crystallographic structure of the eukaryotic yeast 20S proteasome (PDB ID: 1JD2) (Kazi et al., 2003). This type of proteasome structure was also used in this study as it is structurally similar to the mammalian 20S proteasome. Kazi's team reported that the interaction of compound genistein with the proteasomal β5 subunit is responsible for inhibition of the chymotrypsin-like activity. From this study, it was possible to summarise that genistein places the hydroxy group of the B-ring to Thr1 with distance of 1.85 Å, which may sterically block Thr1. Through an analogous protocol in 2004, Smith and friends observed that (−)-EGCG binds to the hydroxy group of the N terminal Thr1 of the proteasome's chymotrypsin-like active site in an orientation and conformation that



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is favourable for inhibition of the proteasomal activity (Smith et al., 2004). Their study also suggested that the lower the docking energy and the larger the cluster, the greater will be the inhibitory potency predicted. Thus, this suggestion has been taken into consideration during the docking analysis of ACA and its analogues with the three distinct proteasomal subunits. The order of the potencies of ACA and its analogues to inhibit the chymotrypsin-like activity of cellular proteasome (Table 4.3) and to increase the expression levels of ubiquitinated proteins (Figure 4.23) in descending manner is AMCA > AEA > ACA. When compared with the experimental data, the order of docking energy of ACA and its analogues to β5 subunit are consistent with the results of the *in vitro* UPS analysis.

Acetoxy group is a common substituent that can be found in ACA, AEA and AMCA. Based on the docking analysis, this substituent helps to bind to the active site of the proteasomal catalytic subunit. Since other functional groups did not exert significant binding to the active site of β subunits, thus there is not much difference between ACA and its analogues in the final docked energy.

The *in silico* docking estimated the internal energy of ligand, docked intermolecular energy and the torsional energy. Among them the intermolecular energy plays a significant role for the final free energy of binding and docked free energy. The intermolecular interactions may be due to the hydrophobic interaction and hydrogen bonding (Kanwar et al., 2010). Thus, acetoxy group is suggested to have more hydrophobic interactions when compared to other structures, with the hydrophobic amino acid residues in the β subunit of proteasome.

Collectively, only the chymotrypsin-like activity and ubiquitination results are consistent with the prediction from the β5 subunit docking analysis.

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## 5.3 ACA and Its Analogues in Relation to the Apoptotic Pathway

Cells undergoing apoptosis can be observed via multiple hallmarks such as the consistent laddering of genomic DNA. In this study, ACA, AEA and AMCA showed active anti-proliferative effects and induced internucleosomal DNA fragmentation on MDA-MB-231 cells, confirming induction of apoptosis. Unlike necrotic cells where DNA is randomly degraded by a range of nucleases, apoptotic cells go through a systematic breakdown of DNA by endonucleases at specific exposed points on the DNA that are not protected by histone protein complexes (Wyllie, 1980). This study showed that DNA fragmentation of cancer cell line following ACA, AEA and AMCA treatment was consistent with MTT assays, where the killing of cells observed were within 24 hrs. During the apoptotic process, proteolytic cleavage of PARP due to activation of caspase-3 (Los et al., 2002), was seen in this study. Previous study showed that ACA induces apoptosis via activation of caspase-3 like activity (Moffatt et al., 2000). Thus, the alteration of PARP by ACA, AEA and AMCA were in agreement with the tendency of apoptotic induction via activation of caspase-3. The activation of p53 protein is also related to apoptosis induction (Geng et al., 2013) and regulation of Bcl-2 family members expression (Sax & El-Deiry, 2003). Anti-apoptotic proteins such as Bcl-2 and Bcl-xL act as the inhibitor of mitochondrial apoptotic pathway, which block the release of cytochromes and counteract the effects of pro-apoptotic proteins such as Bax (Kang & Reynolds, 2009). The pro-apoptotic protein Bax redistributes from the cytosol to mitochondria during apoptotic events (Murphy et al., 2000) to cause dysfunction of the mitochondrial membrane (Wei et al., 2001). It was found in the present study, that the expression of Bax was increased by ACA, AEA and AMCA treatment, while the expression of Bcl-2 and Bcl-xL was decreased. These observations suggest that the induction of apoptosis by ACA, AEA and AMCA was triggered by the upregulation of pro-apoptotic proteins and downregulation of anti-apoptotic proteins which are related to

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the mitochondrial apoptotic pathway. Furthermore, previous study showed that ACA and AEA suppress the growth of cancer cells by inhibiting NF-κB pathway (Ito et al., 2005a; In et al., 2011; Misawa et al., 2015). The expression of the anti-apoptotic proteins such as Bcl-2 (Catz & Johnson, 2001) and Bcl-xL (Tamatani et al., 1999) is regulated by NF-κB. Hence, the induction of apoptosis by ACA, AEA and AMCA may also be linked to the inactivation of the NF-κB.

## 5.4 ACA and Its Analogues in Relation to the Migration Pathway

ACA has also been reported to inhibit metastasis and invasion on different cancer cell types, such as breast cancers (Wang et al., 2014), oral cancers (In et al., 2012), prostate cancers (Pang et al., 2011) and lung cancers (Ichikawa et al., 2005). These discoveries point out that ACA may generally suppress the cell migration and invasion in various cancer cells. In the present study, ACA and its analogues were revealed to exhibit anti-migration effects on MDA-MB-231 cells. Although some of the ACA analogues showed poor potency against cancer cell proliferation, they exhibited strong effects against migration and thus reignites interest for further studies on the molecular mechanisms governing anti-migration activity of ACA and its analogues. Herein, most of the hemi-synthetic ACA analogues were not cytotoxic within the 5.0-50.0μM dose range, but many of them were able to inhibit cancer cell migration with non-toxic concentrations. Hence, the anti-migration activity of ACA and its analogues is not linked to their direct cytotoxic effects on cancer cells. The inhibition of cell migration by the compounds occurred at concentrations that were lower than those that inhibited cell proliferation. The difference in effective concentrations suggested that different mechanisms may be involved to promote ACA and its analogues anti-migratory ability versus cytotoxic effects.

Cancer cell motility by metastasis is a main step for progression of malignancy (Ridley et al., 2003). Tumour cells take multi-step to metastasise from their initial site to

secondary organs by migrating through basement membranes and extracellular matrices (van Zijl et al., 2011). The migration process is stimulated extracellularly and initiated by integrins and intracellular signalling proteins located where the assembly and disassembly of focal adhesions occurs (Golubovskaya et al., 2009). Regulating integrin β1 signalling of focal adhesion kinase (FAK) through modulation of Akt activity could lead to inhibition of cell migration (Choi et al., 2009). Many human malignant cancers show increased FAK expression promoted by integrin β1, resulting in increased metastasis (Zhao & Guan, 2009). Herein, breast cancer cells treated with ACA, AEA and AMCA suppressed integrin β1 after 24 hrs, indicating their ability to interrupt the integrin signalling pathway. Since integrin acts as the activator of FAK, the effect of these compounds on the activation of pFAK was examined. Upon treatment, FAK phosphorylation was significantly reduced in MDA-MB-231 cells. ACA, AEA and AMCA also decreased Akt phosphorylation, showing that activity of pAkt was correlated with FAK phosphorylation. Thus, relative intensities of integrin β1, pFAK/FAK, pAkt/Akt showed that ACA, AEA and AMCA displays anti-migration potency, and is dependent, at least in part, through the integrin β1-mediated signalling pathway.

## 5.5 ACA and Its Analogues in Relation to SAR on Anti-Cancer Effects

To date, little has been known about the SAR of ACA analogues in relation to its anti-cancer effects. Based on experiments involving the use of various chemically modified ACA synthetic analogues, Murakami and friends concluded that a replacement of the 1’-H in ACA by a methyl group resulted in a drastic decrease in cytotoxic activity, and the addition or movement of the phenolic acetoxy group from the *ortho*- to the *para*- and *meta*- positions did not result in an increase in cytotoxicity. It was also suggested that both acetoxy groups in ACA were necessary in cellular permeability properties because ACA analogues with its acetoxy groups replaced with hydroxy groups resulted in the

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reduction of its cytotoxicity (Murakami et al., 2000). It was also reported that the acetoxy groups in ACA were subjected to acetate elimination through hydrolysation by intracellular esterase activity in order to maintain its retention within the cell. As AEA and AMCA possessed similar acetoxy group as ACA, it was also suggested that these two analogues would have undergone similar acetate elimination through hydrolysation esterase activity, resulting in the modified ACA analogues with active biological properties. Xu and friends also reported the structural factors of ACA that regulating tumour cell viability, intracellular GSH level and glutathione reductase activity in Ehrlich ascites tumour cells (Xu et al., 2010). They suggested that the acetoxy group substituted at *para* position at the benzene ring was essential.

In this study, the results showed that the acetoxy group substitute at 4-position and 1'-position are crucial for the anti-proliferative and anti-migration effects. The analogues without the acetoxy group at 4-position (such as analogues **2** and **20**) showed insignificant cytotoxic effects. These findings are consistent with previous studies (Murakami et al., 2000; Xu et al., 2010). Acetoxy groups are electron-withdrawing groups, they would decrease the cationic character of the compounds and increase the rate of hydrolysis and therefore, are able to improve membrane permeability of the compounds (El-Taher, 1996; Xue et al., 2010). This improved membrane permeability property allows ACA and its analogues to diffuse into cells and target specific downstream molecules.

Furthermore, the methoxy group attached at 3-position of the benzene ring is important for both cytotoxic and anti-migration effects. This effect may be due to the fact that methoxy groups are electron-donating groups which facilitate to stabilise the phenoxy radical (Ali et al., 2013). Due to the phenoxy radical stability, methoxy group could help to increase solubility and intracellular retention within the cell, while preventing premature efflux of drugs. However, additional methoxy group at 5-position weakens the

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anti-cancer activities, and this occurrence may be due to the steric hindrance by the extra methoxy group.

On the other hand, hydroxy group bound to the 4-position of benzene eliminate both anti-proliferative and anti-migration activities. The hydroxy group is an electron-donating group and is unable to improve cellular permeability of ACA and its analogues. Hence, reduction of activities was seen in the compound substituted with the hydroxy group.

## 5.6 Future Anti-Cancer Prospects of ACA and Its Analogues

The current study describes the drug development process chain starting from anti-proliferative assays to apoptotic assays, and also elucidates the role of UPS in apoptosis, SAR analysis on anti-cancer effects as well as the regulation of signalling pathways in apoptosis induction and anti-migration activities. As shown in Figure 5.1, it is therefore suggested that ACA, AEA and AMCA possess potential anti-proliferative, pro-apoptotic and anti-metastatic effects, in which apoptosis was mediated via mitochondrial apoptotic pathway and anti-migration via integrin β1-mediated signalling pathway. The knowledge and understanding on how ACA, AEA and AMCA potentiate apoptosis and inhibit migration as shown in this study is of great importance in further understanding the mechanisms underlying tumourigenesis. Moreover, the SAR analysis generated from ACA and its analogues, may then serve as a platform to establish structural requirements for the optimisation of various biologically active compounds. This knowledge will provide the basis for newly targeted therapies, hence giving cancer researchers a better insight for future chemotherapeutic approaches.

The future of ACA, AEA and AMCA is very promising and can be applied greatly in many ways. For example, structural properties of ACA, AEA and AMCA as being a small cell permeable compound, is preferable if comparison to gene transfer strategies in the

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treatment of cancer patients due to its ease in administration. In addition, the ability of ACA, AEA and AMCA to inhibit the expression of cell adhesion proteins in metastatic triple negative breast cancers could therefore interrupt the progression of malignancy, resulting in its reversion to benign tumours.

In brief, the knowledge of mechanism of action of ACA, AEA and AMCA enables development of more efficacious anti-cancer drugs.

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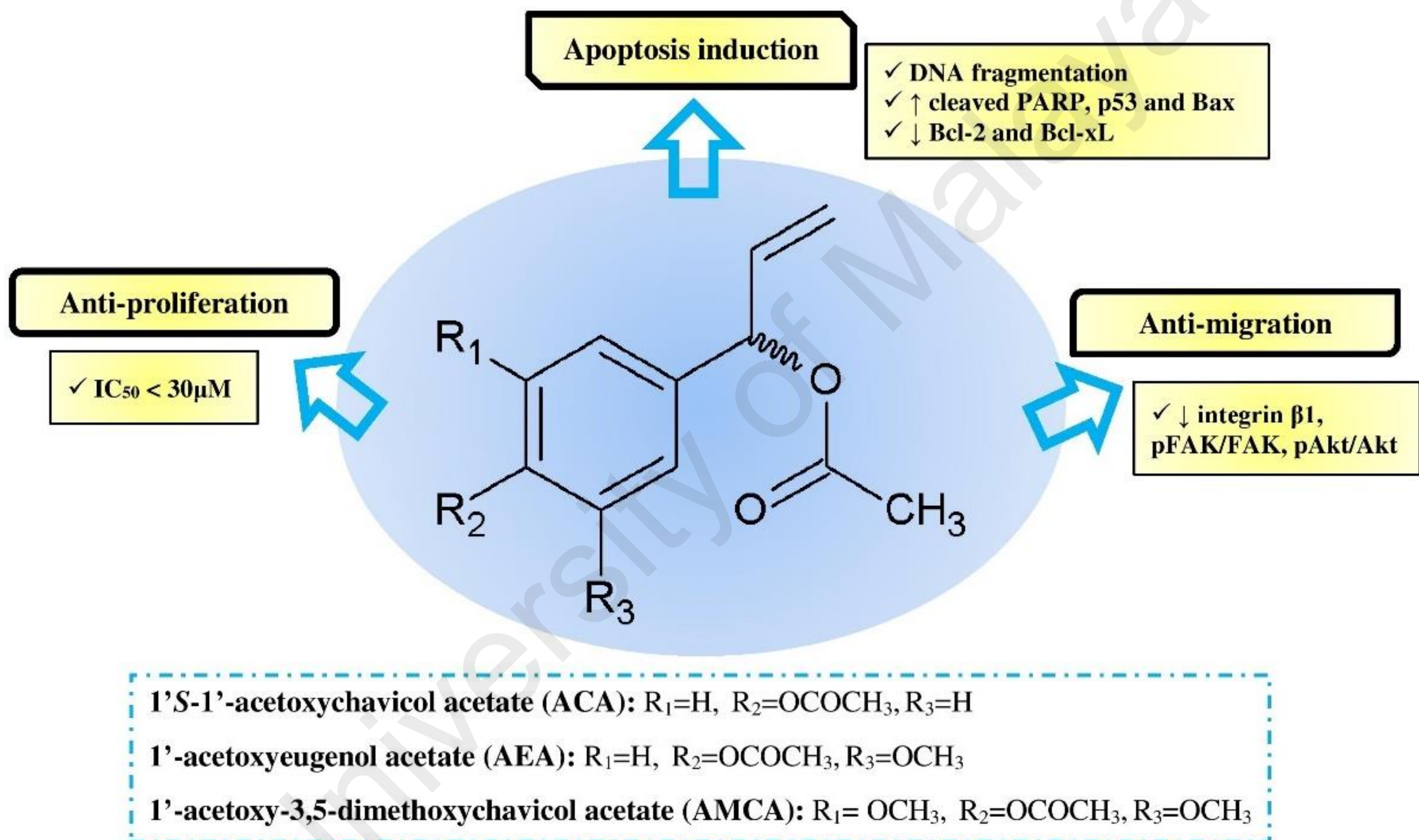


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**Figure 5.1:** Summary of active anti-cancer effects of ACA, AEA and AMCA.

# CHAPTER 6: CONCLUSION

In this study, it has been identified that ACA, AEA and AMCA significantly inhibited growth, induced apoptosis and suppressed migration of human breast cancer cells. Overall, these preliminary results revealed that the anti-proliferative activities are not absolutely required for the suppression of migration in cancer cells by ACA and its analogues. In addition, analogues active in anti-proliferation against selected cancer cells were seen to possess better anti-migration ability against breast cancer cells compared to the natural product itself. Moreover, ACA and its analogues did not regulate the apoptotic induction through ubiquitin-proteasome system. Thus, this study demonstrates that there is still room for improvement in designing new analogues with increased anti-cancer activities. However, more investigations are required to identify the different targets of such ACA analogues at the molecular level and validate the molecular mechanisms in animal models for effective treatment of breast cancer. Overall, this study suggested that ACA, along with its hemi-synthetic analogues, AEA and AMCA, are potential combinatory therapeutic agents for the treatment of triple-negative breast cancers (TNBCs).

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### Publications:

1. **Liew, S. K.**, Azmi, M. N., In, L., Awang, K., & Nagoor, N. H. (2017). Anti-proliferative, apoptotic induction, and anti-migration effects of hemi-synthetic 1'S-1'-acetoxychavicol acetate analogs on MDA-MB-231 breast cancer cells. *Drug Design, Development and Therapy, 11*, 2763-2776.

2. Subramaniam, M., **Liew, S.K.**, In, L.L., Awang, K., Ahmed, N., & Nagoor, N.H. (2018). Inactivation of Nuclear Factor κB by MIP-based drug combinations augments cell death of breast cancer cells. *Drug Design, Development and Therapy, 12*, 1053-1063.

### Conference:

1. ACA and its Analogues: Apoptosis Via the Ubiquitin-proteasome System (UPS). Poster Presentation at the 20th MSMBB Annual Scientific Meeting, 26 June to 27 June 2013, IPPP, University of Malaya, (National).

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