EVALUATION OF *Mycobacterium indicus pranii* AS AN IMMUNO-POTENTIATOR IN COMBINATION WITH 1'S-1'-ACETOXYCHAVICOL ACETATE FROM THE MALAYSIAN *Alpinia conchigera* AND CISPLATIN AGAINST VARIOUS CANCER TYPES

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Field of Study: **Molecular Oncology**

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ABSTRACT

Cancer is a multistage disease consisting of tumour initiation, promotion and progression resulting from the modification of many genes. As a result, in many cases single drug treatment often fails to produce the desired therapeutic effect. In this study, a triple combinatorial usage between the immuno-potentiating activity of *Mycobacterium indicus prani* (MIP), the chemopotentiating properties of 1'S-1'-acetoxychavicol acetate (ACA) from the Malaysian *Alpinia conchigera* and the cytotoxic properties of the commercially available anti-cancer drug, cisplatin (CDDP) was proposed in order to synergistically chemosensitize and eradicate targeted malignancies in anti-cancer chemotherapeutic treatments in both *in vitro* and *in vivo* models. ACA is a phenylpropanoid which is isolated from the rhizomes of a sub-tropical ginger, *Alpinia conchigera*. MIP is a saprophytic bacterium which has been tested in a number of disease models and its immunomodulatory property in leprosy has been well documented. CDDP is a commercial anticancer agent clinically used for the treatment of various malignant tumours, such as head and neck, gastric, bladder, prostate, esophageal and osteosarcoma. In order to identify the potential cytotoxic element(s), a preliminary test was first carried out using four different fractions consisting of live bacteria, culture supernatant, heat killed bacteria and heat killed culture supernatant of MIP against human cancer cells A549 and CaSki by 3-(4,5-dimethyl thiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay. Apoptosis was investigated in MCF-7 and ORL-115 cancer cells by poly-(ADP-ribose) polymerase (PARP) and DNA fragmentation assays. Among the four MIP fractions, only heat killed MIP fraction (HKB) showed significant cytotoxicity in various cancer cells with inhibitory concentration, IC_{50} in the range 5.6–35.0 μl/(1.0×10^6 MIP cells/ml). Evaluation on PARP assay further suggested that cytotoxicity in cancer cells were potentially induced via caspase-mediated apoptosis. The cytotoxic and apoptotic effects of MIP HKB have indicated that this fraction can be a good candidate to further

identify effective anti-cancer agent. In addition, synergistic effects was identified in MCF-7 cells treated with double (MIP/ACA, MIP/CDDP) and triple (MIP/ACA/CDDP) combinations. The type of interaction between drugs/agent was evaluated based on combination index (CI) value being less than 0.8 for synergistic effect. Based on previous studies, mechanism of cell death upon drug combinations which involved intrinsic apoptosis and nuclear factor kappa-B (NF-κB) proteins was validated in western blot analysis. All double and triple combinations confirmed intrinsic apoptosis activation and NF-κB inactivation. Therefore, double and triple combination regimes which targets induction of the same death mechanism with reduced dosage of each drug, is proposed in this study. The *in vitro* combination effects were validated in *in vivo* animal model, BALB/c mice using 4T1 mice breast cancer cells. It was found that mice exposed to combined treatment displayed higher reduction in tumour volume compared to standalone drug. The immunohistochemistry and cytokine analysis provided evidence that combination chemotherapy not only downregulate NF-κB activation, but also reduced the expression of NF-κB regulated genes and inflammatory biomarkers. Consequently, combination therapy shows great therapeutic potential and a pioneer for the basis of future combination drug development.

ABSTRAK

Kanser adalah penyakit berperingkat yang terdiri daripada permulaan, promosi dan perkembangan yang disebabkan oleh modifikasi daripada pelbagai gen. Kesannya pada kebanyakkan masa, rawatan dengan satu ubatan gagal untuk hasilkan kesan terapeutik. Dalam kajian ini, kami mencadangkan penggunaan tiga kombinasi yang terdiri daripada *Mycobacterium indicus prani*, MIP yang meningkatkan imunisasi, 1'S-1'-acetoxychavicol acetate, ACA yang sensitifkan cell dan akhirnya cisplatin, CDDP bekerjasama secara sinergistik untuk membasmi kanser melalui rawatan di luar dan juga dalam badan organisma. ACA berasal daripada tumbuhan *Alpinia conhigera* dan ia adalah fenilpropida yang diambil daripada rizom. MIP adalah bakteria saprofit yang pernah diuji ke atas pelbagai penyakit dan kebolehanya untuk merawat peyakit kusta adalah sangat terperinci. MIP mempunyai peranan penting sebagai vaksin terapeutik yang dibenarkan untuk kegunaan manusia menentang penyakit kusta. Seterusnya, CDDP adalah ubatan anti-kanser komersial yang digunakan secara klinikal untuk merawat pelbagai tumour maliknan. Dalam usaha untuk menemui elemen sitotoksik yang berpotensi, ujian awal dijalankan dengan menggunakan empat pecahan yang terdiri daripada bakteria hidup, supernatan kultur, bakteria mati disebabkan haba dan supernatan kultur mati disebabkan haba daripada MIP ke atas dua kanser sel manusia iaitu, A549 dan CaSki melalui ujian 3-(4,5-dimethyl thiazol)-2,5-diphenyl tetrazolium bromida (MTT). Ujian apoptosis dijalankan ke atas sel-sel MCF-7 dan ORL-115 melalui poly-(ADP-ribose) polymerase (PARP) dan ujian fragmentasi DNA. Daripada empat pecahan, hanya bakteria mati disebabkan haba (HKB) menunjukkan sitotoksik yang signifikan pada pelbagai jenis sel kanser dengan kepekatan inhibitori, IC_{50} dalam lingkungan 5.6–35.0 μl/(1.0×10^6 MIP cells/ml), manakala kesan sitotoksik tidak dapat dikesan pada pecahan lain. HKB tidak menunjukkan kesan sitotoksik pada sel biasa berbanding dengan sel kanser, dan ini menunjukkan kegunaan yang selamat dan keupayannya untuk mengenal

pasti di antara sel biasa dan sel kanser. Evaluasi ke atas ujian PARP menunjukkan sitotoksik dirangsangkan melalui caspase. Kesan sitotoksik dan apoptosis daripada MIP HKB menunjukkan pecahan ini boleh dijadikan sebagai calon yang baik untuk meneruskan pencarian agen anti-kanser yang berkesan. Juga, kombinasi dua (MIP/ACA, MIP/CDDP) dan kombinasi tiga (MIP/ACA/CDDP) antara ACA, MIP dan CDDP menunjukkan hubungan sinergistik apabila diuji ke atas MCF-7 sel kanser payu dara. Hubungan antara dua dan tiga kombinasi ini telah dikenalpasti berdasarkan index kombinasi (CI) dimana CI<0.8 menunjukkan kesan sinergistik. Mekanism kematian sel dikenalpasti berdasarkan kajian lepas merangkumi protein-protein apoptosis intrinsik dan nuclear factor kappaB (NF-κB) yang diuji dengan kaedah pemblotan western. Semua kombinasi disahkan mempunyai apoptosis intrinsik dan pentakaktifan NF-κB di mana keputusan ini menunjukkan kombinasi-kombinasi dua dan tiga menumpukan kepada kematian sel yang sama jenis dengan pengurangan dos. Seterusnya, keputusan ini divalidasi di dalam tikus jenis BALB/c dengan menggunakan 4T1 sel kanser payu dara tikus. Hasil kajian mendapati bahawa tikus yang dirawat secara kombinasi-kombinasi ini telah mempamerkan penurunan dalam pertumbuhan tumor berbanding dengan rawatan secara persendirian. Keputusan imunohistokimia dan sitokin menunjukkan rawatan gabungan yang bukan sahaja mengurangkan ekspresi gen yang dikawal oleh NF-κB tetapi juga mengawal pengurangan ekspresi penanda inflamasi. Oleh yang demikian, rawatan kombinasi menunjukan potensi terapeutik yang tinggi dan menjadi asas perintis bagi perkembangan ubat-ubatan kombinasi.

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LIST OF SYMBOLS AND ABBREVIATIONS

v/v	Volume per volume
w/v	Weight per volume
±SD	Mean standard deviation
x	Times
µM	Micromolar
µL	Microlitre
°C	Degree Celcius
ABC	ATP-binding cassette
ACA	1'S-1'-acetoxychavicol acetate
ADC	Albumin-dextrose complex enrichment
AEA	1'S-1'-acetoxyeugenol
Apaf-1	Apoptotic protease activating factor 1
APS	Antigen presenting cells
AraC	Cytarabine
AVMA	American Veterinary Medical Association
BCG	Bacillus Calmette–Guérin,
BCRP	Breast cancer resistance protein
CC-3	Cleaved caspase 3
CDDP	Cisplatin
CDK4	Cyclin-dependent kinase 4
CD1	Cyclin D1
CI	Combination index
COX-2	Cyclooxygenase-2
CPT-11	Camptothecin-11
CTL	Cytotoxic lymphocytes
CYP	Cytochrome P450
DC	Dendritic cells
DAB	3,3′-Diaminobenzidine
DDR	DNA damage response
DMEM	Dulbecco modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer and xylene
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ER	Estrogen receptor
FASL	Fas ligand
FasL/FasR	Fas Ligand-Fas Receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST	Glutathione-S-transferase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma

HDAC2	Histone deacetylase
HER 2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMEC	Normal human breast cells
H&E	Hematoxylin-eosin
HRP	Horseradish peroxidase
HKB	Heat killed bacteria
HKS	Heat killed supernatant
IFN- γ	Interferon gamma
IGF-1/-2	Insulin-like growth factor-1/-2
IHC	Immunohistochemistry
IKKα/β	IκB kinase alpha/Beta
IL-2	Interleukin 2
IL-12	Interleukin 12
IL-15	Interleukin 15
IC_{50}	50 % Inhibitory Concentration
IC_{25}	25 % Inhibitory Concentration
IC_{10}	10 % Inhibitory Concentration
IKK	IκB kinase
IκBα	Inhibitor of Nuclear Factor Kappa B Alpha
IACUC	Institutional Animal Care and Use Committee
JNK	c-Jun N-terminal kinases
KSFM	Keratinocyte serum-free medium
kDa	Kilo Dalton
KS	Kaposi sarcoma
K-RAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LB	Live bacteria
LS	Live supernatant
MIP	*Mycobacterium indicus pranii*
MIBC	Muscle invasive bladder cancer
MDR1	Multidrug resistance protein 1
MRP1	Multidrug resistance-associated protein 1
MEM-α	Minimum essential medium alpha
MTT	3-(4, 5-dimethylthiazol-2-gl)-2, 5-diphenyl-tetrazoliumbromide
MMP-9	Matrix metallopeptidase-9
NBF	Neutral buffered formalin
NMIBC	Non-muscle invasive bladder cancer
NSCLC	Non-small cell lung cancer
NF-κB	Nuclear factor kappa-B,
NaCl	Sodium chloride
NK	Natural killer
pTEN	Phosphatase and tensin homolog
PR	Progesterone receptor
pRB	Retinoblastoma protein
PBS	Phosphate-buffered saline
PLA2	Phospholipase
P21	Cyclin-dependent kinase inhibitor
Pg/ml	Picograms per millilitre
p-IκB-α	Phosphorylated inhibitor of kappa B
p300	Histone acetyltransferase

PARP	Poly (ADP-ribose) polymerase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RANKL-induced	Receptor activator of nuclear factor-κB ligand
RPMI-1640	Roswell Park Memorial Institute 1640
Rb	Retinoblastoma protein
s.c	Subcutaneous
STAT3	Activators of transcription 3
SCLC	Small cell lung cancer
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TQ	Thymoquinone
TNF	Tumour nuclear factor
TGFβ	Transforming growth factor β
TRAIL	TNF-related apoptosis-inducing ligand
Th1	Type 1 T helper
Th2	Type 2 T helper
TEMED	Tetramethylethylenediamine
TGS	Tris-Glycine-SDS
TBST	Tris Buffered Saline with Tween-20
UGT	Uridine diphospho-glucuronosyltransferase
VEGF	Vascular endothelial growth factor
5-FU	5-fluorouracil

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Globally, incidence of cancers are among the leading causes of morbidity and mortality, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012, and the number of new cases expected to increase to 22 million within the next two decades (Stewart & Wild, 2014). Anti-cancer drugs are an important means to mitigate the impact of cancer mortality. As today, there are many options available to treat cancer. The main types of cancer treatment include surgery, radiation, chemotherapy, immunotherapy, hormone therapy, targeted therapy and many more. The types of treatment is highly dependent on cancer type, cancer stage, possible side effects, level of metastasis, and patients' other health issues.

The mechanisms of cancer development and progression are extremely complex. This is due to the modification of many genes resulting in the alteration of various pathways and biochemical activities in the cells. It is recognized that in many types of cancers there are multiple genetic and epigenetic alterations. Even within a specific cancer type, presence of heterogeneous malignant cell population and diverse genetic changes can be detected over the time due to genetic instability (Pelicano *et al*., 2006). Owing to this, in many cases, single drug treatment often fails to produce the desired therapeutic effect (Humphrey *et al*., 2011). Therefore, to counter the multiple alterations, combination regimens may provide hope for effective cancer treatment.

Combination chemotherapy provides better cure through minimizing metabolic and clinical side effects due to usage of low doses in combination. Next, the cancer adaptation process can be delayed when multiple drugs with different molecular targets are applied, while multiple drugs which targets one single cellular pathway could function

synergistically for both higher therapeutic efficacy and target selectivity (Lee & Nan, 2012). Development of drug resistance in tumour cells can be overcome by using combination drugs (Szakacs *et al.*, 2006). Moreover, the advantage of combinations includes ability of replacing the current most expensive anti-cancer therapies with a much cheaper drug cocktail (Kashif *et al*., 2015). Here, in this study, a natural compound, 1'S-1'-acetoxychavicol acetate, ACA as a chemo-potentiator, *Mycobacterium indicus pranii*, MIP as an immune-potentiator and a commercial drug cisplatin, CDDP will be tested in double and triple combinations in *in vitro* and *in vivo* in various human cancer types.

ACA is a phenylpropanoid which is naturally found in various plant species of the Zingiberaceae family (Khalijah *et al*., 2010). It has been proven to act as an anti-ulceration (Mitsui *et al.*, 1985), anti-allergic (Matsuda *et al.*, 2003), anti-inflammatory (Itokawa *et al*., 1987) and anti-cancer agent (Khalijah *et al*., 2010). ACA was reported to inhibit the constitutive activation of nuclear factor kappa-B, NF-κB through the suppression of IKKα/β activation modulated through dysregulation of the NF-κB pathway (In *et al*., 2012). NF-κB is a family of transcription factors which is constitutively present in cytoplasm and associated with growth and survival of cancer cell (Guttridge *et al.*, 1999). NF-κB controls the expression of over 250 different genes directly and over 400 genes indirectly**.** An over-activation of this pathway observed in most cancer cells causes chemo-resistance and renders cancer cells incapable of undergoing apoptosis. It is therefore hypothesized that a compound which could inactivate or downregulate this pathway, can therefore subsequently enhance the effects of anti-cancer drugs through chemo-sensitization of cancer cells to undergo apoptosis.

CDDP is one of the most widely used anticancer drug against various cancers including sarcoma cancers, cancers of soft tissues, bones, muscles, and blood vessels. Apart

from inducing apoptotic cell death, CDDP is also reported to have direct impact on genomic DNA to form DNA adducts (Andersson *et al.*, 1996) subsequently inhibiting DNA replication and RNA transcription (Ogawa, 1997). Although CDDP has been used in cancer patients for the past 45 years, its efficiency is often accompanied by toxic side effects and tumour resistance, which in turn leads to secondary malignancies (Chen *et al.*, 2009).

The saprophytic bacterium MIP, stimulates cell mediated responses and induces the immune system in patients suffering from a number of diseases like leprosy (Talwar, 1978), HIV (Kharkar, 2002), psoriasis (Rath & Kar, 2003) and cancer specifically bladder and non-small cell lung cancers (Sur & Dastidar, 2003; Chaudhuri & Mukhopadhyay, 2003). The efficacies of MIP in combination with other drugs have not been reported. Since MIP, ACA and CDDP are agents that individually have anti-tumour activity towards cancer, combination of these drugs may translate into improved therapy. Moreover, combining NF-κB inhibitor, in this case ACA with an anti-cancer drug could perhaps enhance overall anti-tumour effect.

Therefore, double and triple combinations regime between these three agents, are tested to identify if there are synergistic chemo-sensitization and eradication of targeted malignancies in anti-cancer chemotherapeutic treatments. MIP, ACA and CDDP as standalone and in combination were treated in various cancer cell lines and their interaction was identified through combination index analysis. Inactivation of NF-κB upon combination therapy was also investigated. *In vivo* animal model study was carried out to investigate and validate combination therapy. In this study, BALB/c female mice were used and treated with various combination regimens subcutaneously. This study also looked into the activation of the immune system upon MIP, ACA and CDDP

administration. This entire study is crucial to address the drug combination effectiveness and their use as potential anti-cancer therapy.

1.1 OBJECTIVES

To investigate the combination use of the immuno-potentiating ability of *Mycobacterium indicus prani* (MIP) with 1'-acetoxychavicol acetate (ACA) from the Malaysian *Alpinia conchigera* and the commercially available anti-cancer drug, cisplatin (CDDP) in order to synergistically eradicate targeted malignancies in anti-cancer chemotherapeutic treatments.

This study embarks with the following objectives:

i) To identify active MIP fraction with cytotoxicity effect.
ii) To investigate cytotoxic and apoptotic effects of MIP on various human cancer cell lines.
iii) To evaluate the synergistic effects of MIP, ACA and CDDP in *in vitro* double and triple combination therapy.
iv) To elucidate the molecular mechanics and apoptotic signalling pathways involved pertaining to the action of MIP in combination with CDDP and ACA, on the NF-κB signalling pathway on selected cancer cell lines.
v) To evaluate the efficacy of combining MIP elements with ACA and CDDP in *in vivo* models.

CHAPTER 2: LITERATURE REVIEW

2.1 Bacteria as an anticancer agent

The role of bacteria as an anti-cancer agent was recognized almost hundred years back by two physicians W. Busch and F. Fehleisen. They separately observed that certain type of cancer regressed following accidental infection with *Streptococcus pyogenes* when patients were hospitalised (Nauts, 1980). Followed by these two German physicians, William Coley separately noticed his patient recovered from neck cancer following the same infection. He then began the very first well documented use of bacteria to treat end stage cancers. He developed a safer therapeutic vaccine made of *S. pyogenes* and *Serratia marcescens* (Richardson *et al*., 1999; Zacharski & Sukhatme, 2005) and it successfully used to treat sarcomas, carcinomas, lymphomas, melanoma and myelomas.

Apart from *Streptococcus* and *Serratia*, other species were also discovered as a potential agent in cancer treatment. Among them, anaerobic bacteria such as *Clostridium*, were able to eliminate cancer cells in the oxygen-poor stage but were harmless to the rest of the body (Malmgren & Flanigan, 1955). Next, bacteria as whole cells can be used as delivery agent for anticancer drugs and as vector for gene therapy (Jain, 2001). For selective tumour destruction, bacteria have been genetically modified. For instance, genetically modified *Salmonella typhimurium* with double auxotrophic leucine-arginine induces regression of pancreatic cancer in orthotopic mice model without the need for any other additional treatment (Nagakura *et al*., 2009).

Bacterial products such as lipopolysaccharides have been tested in cancer treatment and used for tumour destruction which proves that cancer vaccines can be derived from

immunotoxins of bacterial origins (Carswell *et al*., 1975). Bacterial spores can be used as agent to treat cancer as only spores which reach an oxygen starved area of a tumour will germinate, multiply and become active and eventually consume the cancer tissues (Patyar *et al*., 2010).

Next, non-virulent bacteria such as *S. typhimurium* (Avogadri *et al*., 2005), *Clostridium novyi* (Xu *et al*., 2009) and *Bacillus Calmette–Guérin*, BCG (Hayashi *et al*., 2009) are proven to be promising potential immunotherapeutic agents in cancer. Immunotherapeutic approach offers great promise since stimulation of the immune system will destroy cancerous cells by enhancing antigenicity of tumour cells (Xu *et al*., 2009). Figure 2.1 shows overview of these bacterial based approaches.

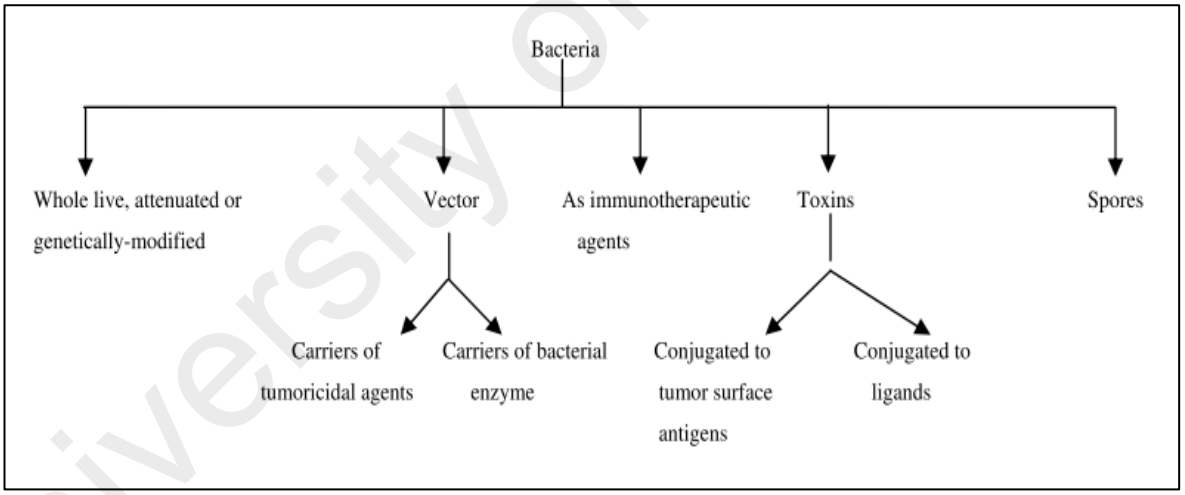


Figure 2.1: Schematic overview of role of bacteria in cancer therapy (Adapted with permission from Patyar *et al*., 2010).

2.1.1 *Mycobacterium indicus pranii* (MIP)

Mycobacterium indicus pranii is a saprophytic soil derived mycobacterial species. This bacterium initially known as *Mycobacterium 'w'*, was listed in Runyon Group IV, along with *M. fortuitum*, *M. smegmatis*, *M. chelonae* and *M. vaccae*, based on its growth and metabolic properties (Zaheer *et al*., 1993). MIP is placed in between the slow and fast growers of the mycobacterial species. It has a growth rate with time of colony appearance approximately 6–8 days that is faster than the typical slow growers, such as, *M. tuberculosis* (~3 weeks) and slower in comparison with typical fast growers, such as, *M. smegmatis* (~3 days) (Saini *et al*., 2009). It appears as a smooth and round colony about 1-2 mm in size on Lowenstein Jensen, Dubos and MB7H11 agar, 5 % NaCl and 10 mg/ml isoniazid at 25 °C to 45 °C (Katoch, 1981). MIP colony does not produce any pigment either in light or dark and it was found to be negative for several tests such as niacin test, Tween-80 hydrolysis as well as for urease test while positive for tellurite reduction.

2.1.2 Immuno-potentiating properties

MIP's immune-potentiating ability is thought to enhance T-helper 1 (Th-1) response resulting in the release of type-1 cytokines, such as, interleukin (IL)-2, IL-12, IL-15 and interferon (IFN)-γ and induce cell-mediated immunity to halt disease progression (Singh *et al*., 1991; Zaheer *et al*., 1995). A model for the mechanism of MIP in cancer study proposed by Rakshit *et al*., (2011) is shown in Figure 2.2. Since it enhances T cell activity, MIP has been used as an immune-adjuvant to chemotherapy for sputum-positive pulmonary TB patients in clinical trials and has resulted in more rapid sputum conversion (Patel *et al*., 2002; Patel & Trapathi, 2003).

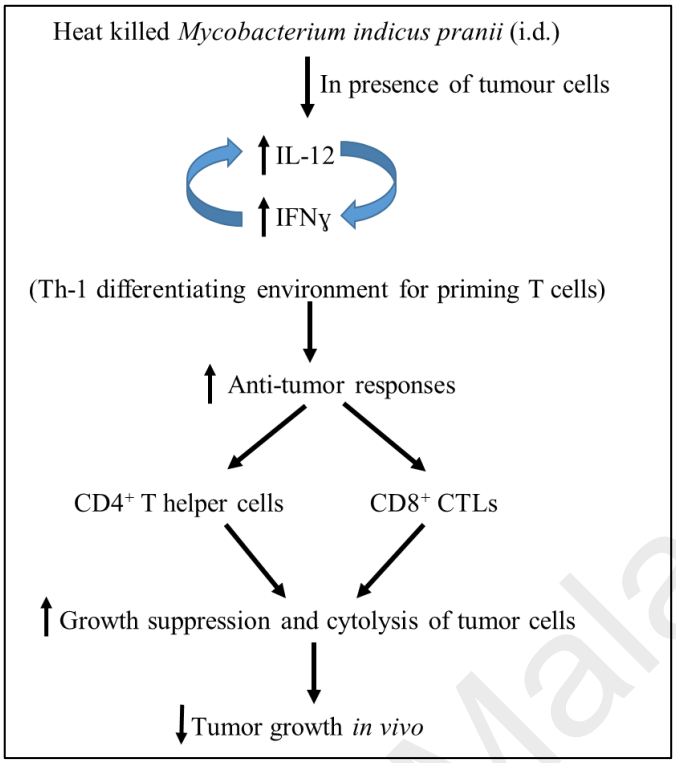


Figure 2.2: A model for the mechanism of MIP mediated anti-tumour response. Treatment of tumour bearing mice with MIP results in high amounts of IL-12 and IFN-γ and increased anti-tumour immune responses mediated by $CD4^+$ and $CD8^+$T cells (Adapted with permission from Rakshit *et al*., 2012).

2.1.3 On-going clinical trial

Based on its demonstrated immunomodulatory action in various human diseases, MIP is the focus of several clinical trials (Table 2.1) and successful completion of one such trial has led to its use as an immunotherapeutic vaccine 'Immuvac' against leprosy (Nath, 1998). MIP has been tested clinically against tuberculosis in 2007 and 2008 in two different clinical trials to study its efficacy as an adjunct therapy in category I pulmonary tuberculosis and in the treatment of type 2 lung tuberculosis patients. Consequently, MIP has been approved to be used in patients with tuberculosis. There are currently on-going pilot trials using MIP on tuberculosis pericarditis. Its immunomodulatory property gained attention in cancer patients when MIP was tested for the first time against superficial transitional cell carcinoma in 2008, achieved a promising outcome.

Tumour regression was observed with intradermal administration of MIP. Following that success, more clinical trials were carried out against prostate cancer, melanoma stage III and IV. In 2008-2010, MIP was tested in combination with other commercial drugs like paclitaxel and cisplatin in advanced non-small cell lung cancer.

Table 2.1: Ongoing clinical trials of MIP in a diverse set of diseases (Adapted with permission from Saini *et al.*, 2012)

Sr. No.	Duration	Diseases	Objective of ongoing trials	Phase	Trial No.	Intervention
1	2007–09	Tuberculosis	Efficacy and safety of immunomodulator (*MIP*) as an adjunct therapy in Category I pulmonary tuberculosis along with assessment of immunological parameters	III	NCT00341328	*MIP* alone and also along with Category I ATT drugs as per RNTCP guidelines
2	2008–10	Tuberculosis	To study the efficacy and safety of *MIP* in the retreatment of lung (Type 2) tuberculosis patients	III	NCT00265226	Intra-dermal administration of *MIP*
3	2008–11	Tuberculous pericarditis	A pilot trial of adjunctive prednisolone and *MIP* with immunotherapy in tuberculous pericarditis	III	NCT00810849	Prednisolone and *MIP* immunotherapy
4	2008–11	Superficial transitional cell carcinoma	To compare the efficacy, toxicity and time to tumor regression by treatment with *MIP* (intra-dermal) and BCG (intravesical) in patients with newly diagnosed STCC with high probability of recurrence	II	NCT00694915	*MIP*, BCG
5	2007–10	Hormone refractory prostate cancer (HRPC)	To compare the overall survival, hematological toxicity, pain reduction score, response to tumor, quality of life in two arms of HRPC patients from different parts of India	II	NCT00525408	*MIP* as an adjuvant to docetaxel
6	2008–10	Superficial transitional cell carcinoma	To evaluate the response rate of *MIP* treatment in patients, detecting its effect on time to tumor progression and evaluating its safety	I	NCT00694798	*MIP*
7	2006–10	Stage III or Stage IV melanoma	To evaluate clinical response, immune response and safety of treating patients with advanced stage melanoma with the vaccine CADI-05	I, II	NCT00675727	*MIP*
8	2008–10	Non-small cell lung cancer	To determine efficacy of *MIP* in combination with paclitaxel plus cisplatin in advanced non-small cell lung cancer	II	NCT00680940	*MIP*, paclitaxel and cisplatin

2.2 Cisplatin (CDDP)

Cisplatin, *cis*-[Pt(II) (NH_3)$_2$$Cl_2$)] ([$PtCl_2(NH_3)_2$] or CDDP is a platinum based chemotherapy drugs widely used for cancer treatment. CDDP was first synthesized in 1845 and later in 1893, its structure was deduced by Alfred Werner (Desoize & Madoulet, 2002). Its usage in cancer treatment started in 1971 and became available for clinical practice in 1978, as Platinol® (Bristol-Myers Squibb). Different type of cancer like sarcoma, cancers of soft tissue, bones, muscles, and blood vessels has been successfully treated by CDDP. Clinical success of CDDP gives room for developing other effective metal-based anti-cancer compounds like palladium and nickel (Chen *et al*., 2009b; Frezza *et al*., 2010; Che & Siu, 2010). The mode of action of this platinum drug is via inhibition of DNA synthesis and repair. It damages tumours through activation of apoptosis through various signal transduction pathways (Figure 2.3).

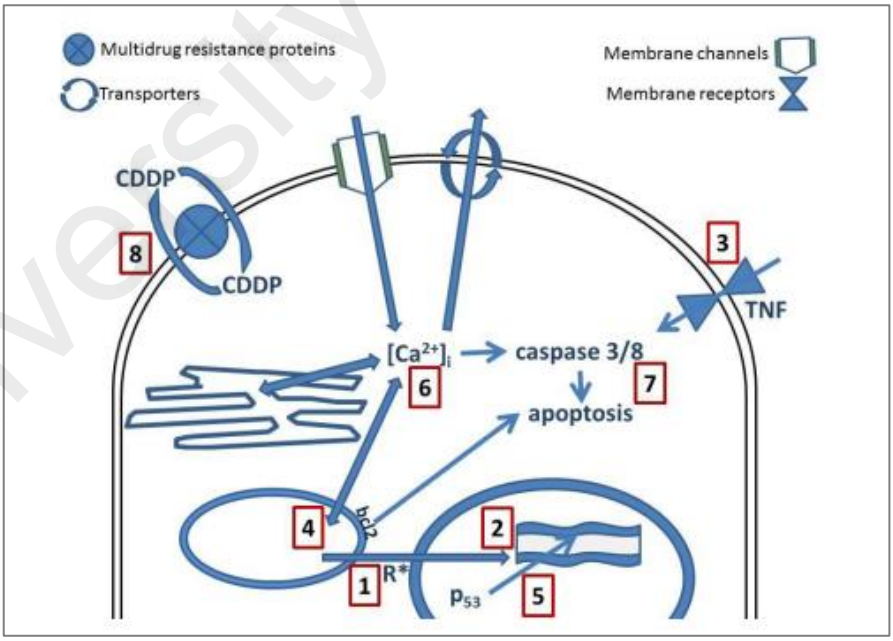


Figure 2.3: Cellular interactions of CDDP: (1) reactive oxygen species; (2) DNA; (3) TNF; (4) mitochondria; (5) p53; (6) calcium signaling; (7) caspases; (8) multidrug resistant proteins. (Adapted with permission from Florea & Büsselberg, 2011).

Despite the positive effects of platinum compounds, they are highly toxic. Side effects of platinum therapy include general cell-damaging effects, such as nausea and vomiting, decreased blood cell and platelet production in bone marrow (myelosuppression), decreased response to infection (immunosuppression), nephrotoxicity, neurotoxicity and ototoxicity (Page & Takimoto, 2004). Just like many other anti-cancer agents, resistance towards cisplatin has been reported where cells failed to undergo apoptosis at clinically relevant doses or at achievable plasma drug concentrations. Studies have also shown that different resistance mechanisms exist between different cell lines (Teicher *et al*., 1990; Kelland, 1993). Among the mechanisms which have been found to contribute to cisplatin resistance are reduced drug uptake, enhanced drug efflux, increased inactivation by thiol-containing molecules, enhanced DNA damage repair, overexpression of HER-2/Neu and PI3K/AKT as well as Ras/MAPK pathway, loss of p53 tumour suppressor function and inhibition of apoptosis (Siddik, 2003).

2.3 Natural compounds as anti-cancer agents

Natural products are valuable resources originated from plant, microbes and marine that provides a variety of bioactive compound in modern drug discovery. For over 40 years, natural products have established as cancer chemotherapeutic agents either in unmodified (natural) form or synthetic, modified form (Kinghorn, 2008). These include compounds from plants (such as elliptinium, galantamine and huperzine), microbes (daptomycin) and animals (exenatide and ziconotide), as well as synthetic or semi-synthetic compounds based on natural products (e.g. tigecycline, everolimus, telithromycin, micafungin and caspofungin). Over a 100 natural-product-derived compounds are currently undergoing clinical trials and at least a 100 similar projects are in preclinical development (Table 2.2).

Table 2.2: Drug based natural products as different stages of development. Natural products obtained from plant, bacterial, fungal, animal and semisynthetic. (Adapted with permission from Harvey, 2008)

Drugs based on natural products at different stages of development						
Development stage	Plant	Bacterial	Fungal	Animal	Semi-synthetic	Total[a]
Preclinical	46	12	7	7	27	99
Phase I	14	5	0	3	8	30
Phase II	41	4	0	10	11	66
Phase III	5	4	0	4	13	26
Pre-registration	2	0	0	0	2	4
Total	108	25	7	24	61	225

2.3.1 Plant derived natural compounds

Many FDA approved plant derived compounds have been successfully employed in cancer treatment. The search in 1950s, started with the discovery of vinca alkaloids and isolation of the cytotoxic podophyllotoxins. Vinca alkaloids were isolated from periwinkle, *Catharanthus roseus* or vinca rosea from the rainforest of Madagascar. These alkaloids and its semisynthetic derivatives induce cell apoptosis by blocking mitosis at the metaphase stage. They have since been used in the treatment of lymphomas, Kaposi sarcoma (KS) and testicular, breast and lung cancers (Heijdren *et al*., 2004; Balunas & Kinghorn, 2005).

The next widely used chemotherapeutic agents are from class of molecules called taxanes such as paclitaxel, taxol® and its derivative docetaxel, Taxotene®, which were isolated from the bark of *Taxus brevifolia* Nutt, Taxaceae. Paclitaxel has been used in treatment of breast, ovarian, and non-small cell lung cancer (NSCLC), and has also shown efficacy against Kaposi sarcoma, while docetaxel is primarily used in the treatment of breast cancer and NSCLC.

There are also many in clinical trials, one of the most important being curcumin, a natural compound isolated from different *Curcuma* species, zingiberaceae. Curcumin has proven to have anti-angiogenic effects in a diverse number of animal and cell-culture models (Wang *et al*., 2015). It inhibits 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced inflammation (Garg *et al*., 2008), hyperplasia, proliferation, ornithine decarboxylase (ODC) (Kunnumakkara *et al*., 2008), reactive oxygen species (ROS) generation, COX, and lipoxygenase in mice (Kohli *et al*., 2005). Curcumin inhibits the carcinogenesis process in animal model by suppressing the promotion and progression stages of cancer development (Kuttan *et al*., 1987; Rao *et al*., 1995).

Resveratrol is a naturally occurring polyhydroxylated stilbene found in grapes, red wines and mulberries which were proven to have protective effects against carcinogenesis apart from its antioxidant property (Aziz *et al*., 2005; Pezzuto, 2008). Resveratrol was effective against a number of cancers including the tumours of pancrease (Ding & Adrian, 2002), breast (Banerjee *et al*., 2002), gastrointestinal tract (Sun *et al*., 2002), lung and soft tissues (Kubota *et al*., 2003), prostate (Gill *et al*., 2007) and liver (Khan *et al*., 2008).

Thymoquinone (TQ) is the bioactive compound of the volatile oil of black seed (54 %) and was first extracted by El-Dakhakhany (1963). Growth inhibitory effects of TQ are specific to cancer cells (Gali-Muhtasib *et al*., 2004). It showed significant anti-neoplastic activity against human pancreatic adenocarcinoma cells by inhibiting NF-κB activation (Worthen *et al*., 1998). TQ has been shown to induce apoptosis by p53-dependent (Gali-Muhtasib *et al*., 2004) and p53-independent (El-Mahdy *et al*., 2005) pathways. TQ also exerts anti-oxidant effects and inhibits inflammation in animal models and cell culture systems (Houghton *et al*., 1995; Mansour *et al*., 2002).

Other natural metabolites, such as, flavanoids and phenolic compounds also plays important role in anti-cancer drug identification. They were found to have pleiotropic influence on cellular signaling by the inhibition of transcription factors, such as, NF-κB or Nrf2 (Rahman *et al.*, 2006; Prasad *et al.*, 2010), or antioxidative effects (Pietta, 2000; Rahman *et al.*, 2006). Furthermore, polyphenols are found in high concentrations in many fruits and vegetables, resulting in a continuous and long-term intake of such plant phenols.

2.3.2 Mechanism of natural chemo-preventive compounds

Natural compound-based drugs are designed to be multi-targeted, to eradicate cancer cells which had already went through multiple mutations. Among all drug targets, transcription factors NF-κB and STAT3 which regulates the expression of more than 400 gene products appear to be vital for both prevention and cancer treatment (Ahn & Aggarwal, 2005; Sethi *et al*., 2008; Aggarwal *et al*., 2009). They control important processes in cancer cells such as transformation of normal cell to cancer cell, survival, proliferation, invasion, angiogenesis, epithelial–mesenchymal transition (EMT), and metastasis. NF-κB is present in constitutively active form in tumour cells, however most cancer risk factors such as stress, alcohol, tobacco, radiation, infectious agents (viruses) and growth factors receptors, such as, EGFR, HER2, TNF receptors have been shown to activate NF-κB. Thus, ultimately suppression of NF-κB and STAT3 pathways are ideal strategy for both prevention and treatment of cancer (Luqman & Pezzuto, 2010). Aggarwal and coworkers have shown that natural compounds, such as, curcumin, capsaicin derived from red chili, TQ-derived black cumin, anethole from fennel, eugenol from cloves, and zerumbone derived from ginger are able to suppress both NF-κB and STAT3 pathways in a variety of tumour cells leading to inhibition of tumour cell survival, proliferation and invasion. Moreover, these agents are also able to sensitize tumours to radiation (Sandur *et al*., 2009)

and chemotherapeutic agents (Ye *at al*., 2007), thus has the potential to be used not only for prevention but also for treatment. The cell signaling pathways activated by natural dietary agents are diverse. Moreover, the same compound may activate different signaling pathways on different cell types. Some of the important signaling pathways targeted by botanicals are shown in Figure 2.4 and described below.

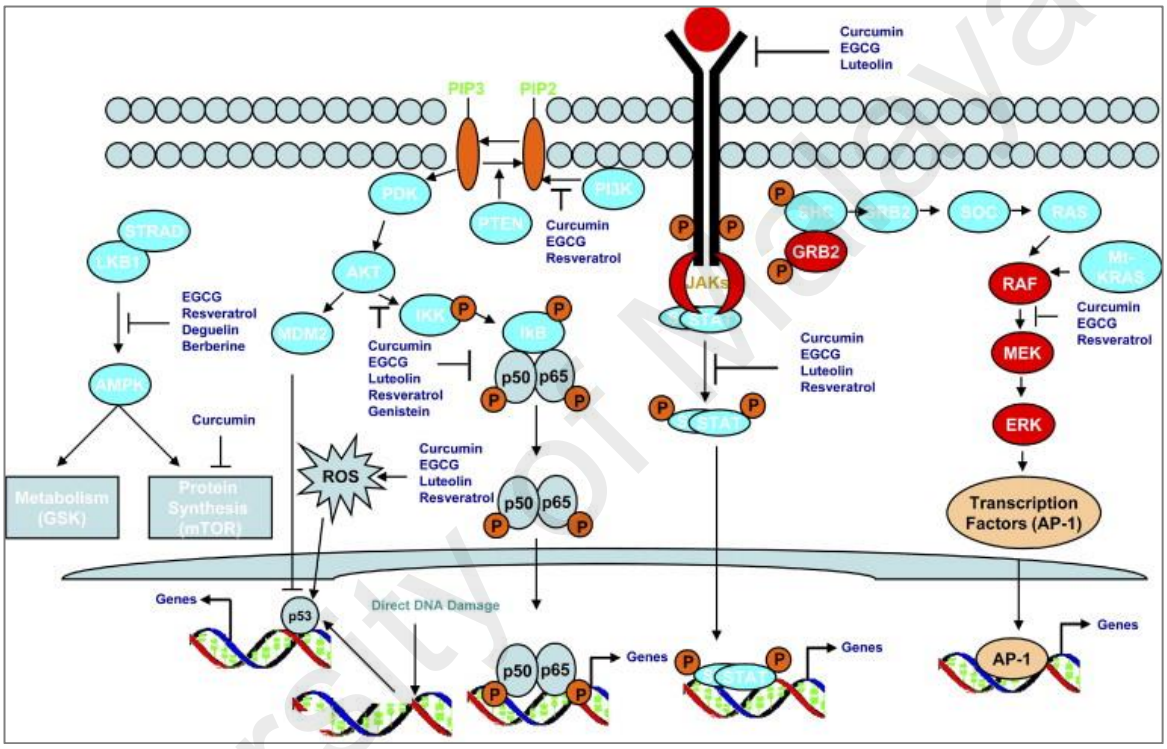


Figure 2.4: Some of the important molecular pathways affected by phytochemicals.

2.3.3 *Alpinia conchigera* Griff

Alpinia conchigera Griff is known locally as lengkuas ranting, lengkuas kecil, lengkuas padang, lengkuas geting or chengkenam (Burkill, 1966; Janssen & Scheffar, 1985; Kress *et al*., 2005). The plant is an herbaceous perennial, 2-5 ft tall, and found in Eastern Bengal and Peninsular Malaysia and Sumatera (Larsen *et al*., 1999). The plant grouped under Zingiberaceae family, section: Zingiberacea and sub-section: Strobidia. Locally, it is used as condiment in the northern state of Peninsular Malaysia and occasionally in traditional

medicine to treat fungal infection (Ibrahim *et al*., 2000) while in Thailand, the rhizomes are used in traditional Thai medicines to treat gastrointestinal disorders and in the preparation of Thai food dishes (Matsuda *et al*., 2005).

A study by Hasima *et al*. (2010) successfully isolated two experimentally active compounds from the plant *Alpinia conchigera*, namely, 1'S-1'-acetoxychavicol (ACA) and its analog 1'S-1'-acetoxyeugenol (AEA). These compounds demonstrated cytotoxic and apoptotic effects towards MCF-7 breast cancer cells growth. On top of that, ACA has been identified as the major cytotoxic component in Alpinia species following bioassay-guided fractionation from two sub-species i.e. *Alpinia galanga* and *Alpinia officinarum* (Lee & Houghton, 2005).

2.3.4 ACA

1'S-1'-acetoxychavicol acetate (ACA), is a natural phenylpropanoid (Figure 2.5) found in various ginger species worldwide. ACA has been associated with a number of various medicinal properties including anti-ulceration (Mitsui *et al*., 1985), anti-allergic (Matsuda *et al*., 2003), anti-inflammatory and anti-cancer activities (Itokawa *et al*., 1987; Khalijah *et al*., 2010). Previous studies have shown that ACA has anti-inflammatory and anti-oxidant properties by suppression of xanthase oxidase activity (Ohnishi *et al*., 1996), superoxide anion generation (Murakami *et al*., 1996) and inducible nitric oxide synthase expression (Ohata *et al*., 1998).

ACA has also been shown to exhibit chemo-preventive activity against various cancer type as single or combination agent. ACA induces tumour apoptosis and tumour-related inflammation in glioblastoma cells by inducing caspase 3 activity (Williams *et al*., 2013). Other reports include the involvement of ACA in apoptosis induction through stimulation

of caspase-8 and -9 in myeloid leukemia cells via mitochondrial and Fas-mediated dual mechanism (Ito *et al*., 2004). ACA has been shown to act as a potential NF-κB inhibitor by blocking the IκBα kinase activity (Ichikawa *et al*., 2005) and by inhibiting RANKL-induced NF-κB activation (Ichikawa *et al*., 2006). In a different study, effects of ACA correlated with inhibition of NF-κB regulated genes (FASL and BIM), including pro-inflammatory (COX-2) and proliferative (cyclin D1) biomarkers in tumour tissues (In *et al*., 2012). In combination treatment, ACA further enhanced cytotoxic effects of cisplatin (CDDP) in a synergistic manner which produces an improved chemotherapeutic regime with increased efficacies at lower concentrations, by reducing the occurrence of dose-limiting toxicities (In *et al*., 2012; Phuah *et al*., 2012).

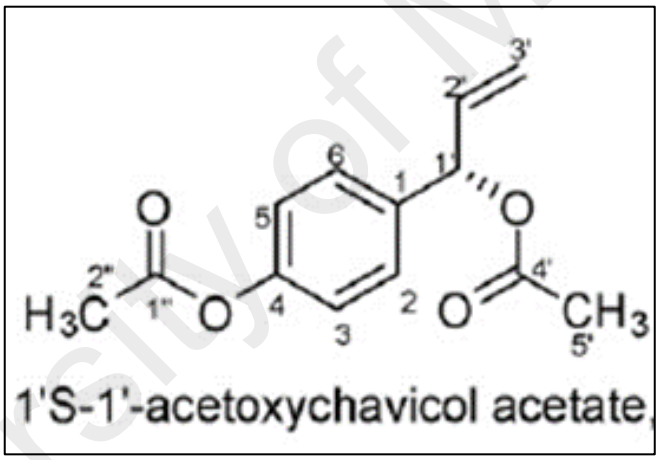


Figure 2.5: Chemical structure of 1'S-1'-acetoxychavicol acetate

2.4 Cell death

Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or of distinct classes of proteases, such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics like

immunogenic or non-immunogenic (Melino, 2001). Table 2.3 shows distinct modalities of cell death.

Table 2.3: Distinct modalities of cell death (Adapted with permission from Kroemer *et al*., 2009)

Cell death mode	Morphological features
Apoptosis	Rounding-up of the cell Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes, *in vivo*
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells, *in vivo*
Cornification	Elimination of cytosolic organelles Modifications of plasma membrane Accumulation of lipids in F and L granules Extrusion of lipids in the extracellular space Desquamation (loss of corneocytes) by protease activation
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation

2.4.1 Apoptosis

Apoptosis is a biochemical event involving rounding up of the cell, retraction of pseudopods, reduction of cellular volume known as pyknosis, chromatin condensation, nuclear fragmentation and plasma membrane blebbing (Kroemer *et al*., 2008). Apoptosis with similar morphological changes can be triggered through two different pathways, namely, intrinsic and extrinsic with or without involvement of mitochondria, respectively (Danial & Korsmeyer, 2004; Kroemer *et al*., 2007).

However, these two pathways are linked and molecules in one pathway can influence the other pathway (Igney & Krammer, 2002). There is in addition a pathway in apoptosis comprising of T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell via either granzyme A or B. All three pathways share the same execution pathway through cleavage of caspase-3 and subsequently resulting in DNA fragmentation, cross linking of proteins and formation of apoptotic bodies (Figure 2.6). However, granzyme B has a slightly different path where it activates a parallel, caspase-independent cell death process (Martinvalet *et al*., 2005).

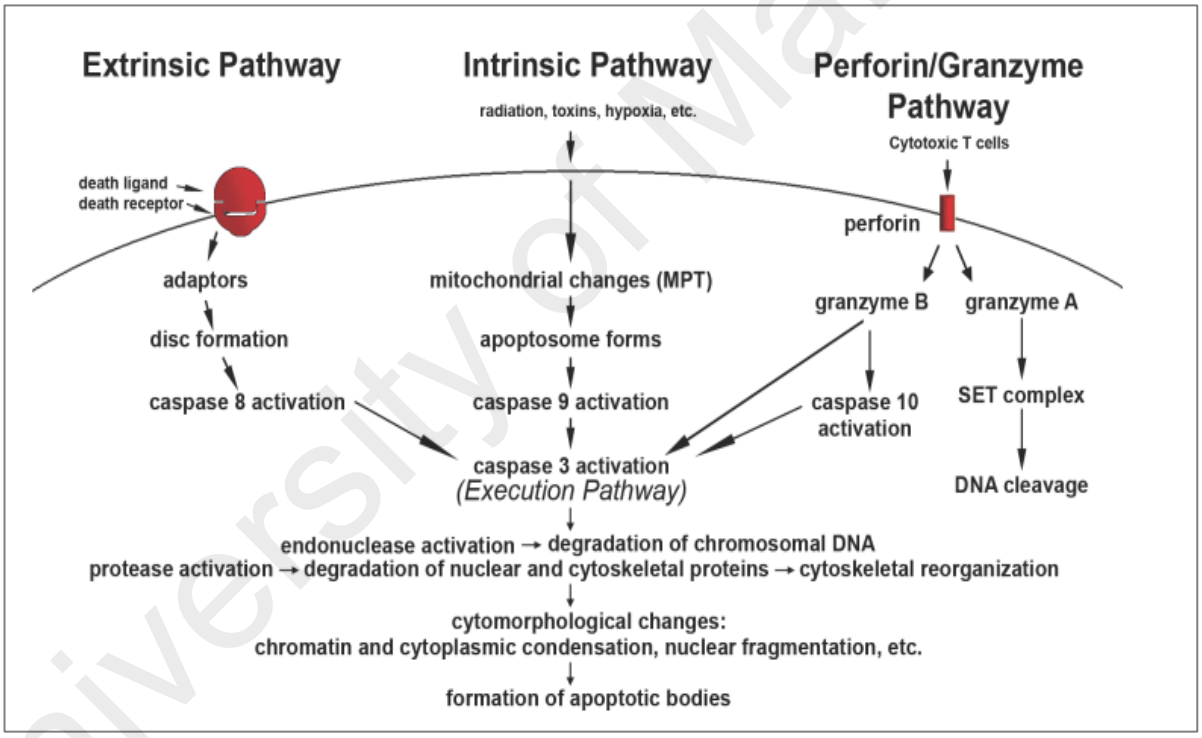


Figure 2.6: Schematic representation of apoptotic events. The two main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/ granzyme pathway. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cyto-morphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages.

2.4.2 Intrinsic pathway

Intrinsic signalling pathway is a mitochondrial initiated event that acts directly on targets within the cells. The stimuli that initiates intrinsic pathway are capable of creating either positive or negative intracellular signals. Negative signals can lead to failure in suppression of cell death due to absence of growth factors, hormones and cytokines, thus leading to apoptosis. While, other signals that act in positive way are through radiation, toxins, viral infection and hypoxia. Both these signals affects inner mitochondrial membrane where opening of pores occurs, loss of transmembrane potential and release of pro-apoptotic protein (cytochrome-c) from inter-membrane space into the cytosol. Cytochrome-c binds to both Apaf-1 and pro-caspase-9 to form ''apoptosome'' (Chinnaiyan, 1999; Hill *et al*., 2004) to activate caspase 9.

2.4.3 Extrinsic pathway

Apoptotic extrinsic pathway involves transmembrane receptor-mediated interactions which involves death receptors that are members of tumour nuclear factor (TNF) receptor gene superfamily (Locksley *et al*., 2001). Members of TNF receptor family, shares similar death domain which plays critical role in directing the death signal from the cell surface to the intracellular signaling pathways. FasL/FasR, TNF-alpha/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo/DR5 are among the more common ligands corresponding to death receptors (Chicheportiche *et al*., 1997; Ashkenazi *et al*., 1998; Peter & Kramer, 1998; Rubio-Moscardo *et al*., 2005).

2.4.4 Perforin/granzyme pathway

Cytotoxic T-cells control the perforin or granzyme pathway in order to induce cytotoxicity. Perforin, a transmembrane pore-forming molecule, activates this pathway with a subsequent exophytic release of cytochrome granules containing granzyme A and B through the pore into the target cells (Trapani & Smyth, 2002). Granzyme A activates caspase-independent cell death via DNA damage while mitochondrial pathway is activated by granzyme B (Goping *et al*., 2005).

2.4.5 Role of apoptosis in cancer

Rate of cell proliferation and cell-cell communication or attrition contributes to cell division thus increased cell population occurs. Apoptosis represents the major source of cell attrition, especially via extrinsic and intrinsic pathways. In many cancer types, mutations in these two pathways lead to resistance towards apoptosis (Hanahan & Weinberg, 2011). The possibility of apoptosis being a barrier to cancer was identified, when it was reported that increased apoptosis occurred in fast growing and hormone dependent cells following hormone withdrawal (Kerr e*t al*., 1972).

During tumour development, a variety of signals plays key role to trigger apoptosis. Studies using transgenic and knockout mice provide direct evidence that disruption of apoptosis can promote tumour development. Loss of p53, a tumour suppresser gene by gene 'knockout' accelerates tumour progression in murine retina, lens, choroid plexus and in the lymphoid compartment (Attardi & Jacks, 1999). Additionally, crucial role of extracellular survival factors, such as, insulin-like growth factor-1/-2 (IGF-1/-2) and IL-3 in supporting tumour progression was revealed in mouse studies where tumour from IGF-2 animals remain hyperplastic and showed excessive apoptosis (Christofori *et al.*,

1994). Besides this, intracellular signals from Ras, and loss of pTEN expression can activate anti-apoptotic signals and allows cancer cells to evade apoptosis (Evan & Littlewood, 1998). Disruption of apoptosis may also contribute to tumour metastasis.

The interconnected signalling that controls apoptosis has revealed how apoptosis is triggered in response to various stress signals in cells and this apoptotic circuitry is often attenuated at some point in tumour, enabling them to progress to high-grade malignancy (Adams & Cory, 2007). The understanding of this interconnection, has allowed for it to be used in cancer treatments to repair the faulty apoptotic programs in cancer cells by inducing apoptosis through manipulation in the expression of genes involved in apoptosis regulation.

2.5 Cancer overview

Continuous unregulated proliferation of cells results in cancer development. Unfortunately, signal that controls normal cells fail to work in cancer cells, thus they grow and divide in an uncontrolled manner then invading normal tissues and organs and eventually spreading throughout body.

Cluster of proliferated cells form tumour, which may be either benign or malignant. Benign tumour, remains confined to its original locations without invading surrounding normal tissues or organ or spreading to distant body sites. A malignant tumour on the other hand is capable of invading surrounding tissues and spreading throughout the body via circulatory or lymphatic system. A malignant tumour is termed as cancer and is dangerous and life threatening. Benign tumours are not as dangerous as malignant cancer because it can usually be removed through surgery.

Cancers categorized into four main groups: carcinomas, sarcomas, leukemia/lymphomas and neuroectodermal according to the type of cells from which they arise. Carcinomas made up 90% of human cancers and are malignancies of epithelial cells. Sarcomas, rare in human, are solid tumours of connective tissues, such as, bone, muscle, cartilage and fibrous tissue. Leukemia/lymphomas arise from the blood-forming cells and cells of the immune system which account for 8 % of human malignancies. The last group arises from cells that form various components of the central and peripheral nervous system termed neuroectodermal. Tumours are further classified according to tissue of origin for example fibrosarcomas arise from fibroblast, and erythroid leukemia from red blood cells (Cooper, 2000).

2.5.1 Breast cancer

Breast cancer is the most frequent malignancy in female. To date, U.S. Breast Cancer statistics shows about 12 % U.S. women are affected by invasive breast cancer over the course of their lifetime. In 2017, 255,180 new cases of invasive and 63,410 of non-invasive breast cancer cases are expected. In Malaysia, about 1 in 19 women in this country are at risk, compared to 1 in 8 in Europe and the United States. The main cause of death due to breast cancer is because of its tendency to metastases at distant site such as lymph nodes, bone, lung, liver and brain. Hormone exposure, family genotypes, alcohol consumption, early menarche, late menopause, low or late parity and post-menopausal obesity are all found to be established risk factors of breast cancer (Kolonel *et al*., 2004).

Cancer cells heterogeneity are identified via histopathology by observing morphology and are further confirmed through molecular profiling techniques, such as, DNA

microarray. Classification of breast cancer cells are based on several criteria like histological type, tumour grade, lymph node status and presence of markers, such as, estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). Based on this, breast cancer can be classified into five subtypes: luminal A, luminal B, HER2, basal and claudin-low (Perou *et al*., 1999) as shown in Table 2.4.

Table 2.4: Molecular classification of breast carcinoma (Adapted with permission from Perou *et al*., 1999)

Classification	Immuno-profile	Other characteristics	Example cell lines
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	Ki67 low, endocrine responsive, often chemotherapy responsive	MCF-7, T47D, SUM185
Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	Ki67 high, usually endocrine responsive, variable to chemotherapy. $HER2^+$ are trastusumab responsive	BT474, ZR-75
Basal	ER^-, PR^-, $HER2^-$	$EGFR^+$ and/or cytokeratin $5/6^+$, Ki67 high, endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468, SUM190
Claudin-low	ER^-, PR^-, $HER2^-$	Ki67, E-cadherin, claudin-3, claudinin-4 and claudinin-7 low. Intermediate response to chemotherapy	BT549, MDA-MB-231, Hs578T, SUM1315
HER2	ER^-, PR^-, $HER2^+$	Ki67 high, trastusumab responsive, chemotherapy responsive	SKBR3, MDA-MB-453

EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

BT-20 was the first breast cancer cell established in 1958 (Lasfargues & Ozzello, 1958), followed 20 years later by M.D. Anderson series such as MDA-MB-231 and MDA-MB-436 (Cailleau *et al*., 1978). The cancer cell line MDA-MB-231, was isolated from pleural effusions of a 51 years old Caucasian breast cancer patient. This cell line lack functional receptors like estrogen and progesterone or HER2. They are known as triple negative cell line and constitute as a representative model for chemotherapy studies. Triple negative breast cancer cells remain the hardest cancer subtype to treat because it is an assemblage

of different breast cancer subtypes and an aggressive tumour which has metastases at an early stage (Kumar *et al*., 2013). The cancer cell line, MCF-7 established in 1973 at the Michigan Cancer Foundation, still remains as the most popular breast cancer cell line in the world (Soule *et al*., 1973; Kern *et al*., 1994). This cell line, with an epithelial like morphological appearance, was first established in 1970 from pleural effusion of the mammary gland of a 69 years old female Caucasian (Soule *et al*., 1973). MCF-7 was found to be non-invasive, express relatively high amount of IGF-IR (Dickson *et al*., 1986), tested positive for E-cadherin expression (Hiraguri *et al*., 1998), contain epidermal growth factor receptors (Biscardi *et al*., 1998) and progesterone receptors (Sutherland *et al*., 1988). Therefore, MCF-7 is a perfect model for hormone therapy studies.

2.5.2 Liver cancer

Liver cancer is more common in Sub-Saharan Africa and Southeast Asia than in the U.S. More than 700,000 people are diagnosed with this cancer each year throughout the world. It is also the leading cause of cancer death worldwide and according to American Cancer Society (2016) approximately more than 600,000 deaths are recorded each year. Among the risk factors identified for liver cancer cases are hepatitis B virus (HBV), hepatitis C virus (HCV), dietary aflatoxins, alcoholic liver diseases and hemochromatosis (Kamangar *et al*., 2006).

HepG2 is a human liver carcinoma cell line derived from a liver biopsy of a 15 year old Caucasian male. It is a well differentiated hepatocellular carcinoma (HCC) with epithelial-like morphology. HepG2 cells were reported to produce several cellular products, such as, complement (C4), C3 activator, fibrinogen, albumin, alpha-fetoprotein (Knowles *et al*., 1980), HMG-CoA reductase and H-TGL activities (Busch *et al*., 1990).

There have been no reports that indicated the presence of any HBV surface antigens or HBV sequences in the genome of this cell line (Aden *et al*., 1979). However, insulin-like growth factor II (IGF-II) receptors have been detected on HepG2 cells (Schardt *et al*., 1993).

2.5.8 Cervical cancer

Cervical cancer is a cancer malignant of the cervix or within the cervical area. The cervix is the lower part of uterus that connects the uterus and vagina. Most cervical cancer begin in the transformation zone, where endo-cervix and exo-cervix meet since this zone is less stable and more susceptible to viral infections (American Cancer Society, 2010). Majority of cervical cancer are squamous cell carcinomas (80-90 %) while, the rest are adenocarcinomas which develops from the mucus-producing gland cells. Cervical cancer is one of the deadliest cancers of women, but easily preventable with early detection of regular screening. Screening test such as pap-smear is used to identify pre-cancers, which can be treated to prevent cancer mortality. Cervical cancer causes 270,000 deaths annually of which 85 % occurs in developing countries (Kumar, 2016). In 2012, it was the fourth most commonly diagnosed cancer with an estimated 527,600 new cases worldwide. The number of cervical cancer cases is expected to increase 1.5 fold by 2030 with an increase in population and aging. In Malaysia, it ranks as the second leading cause of female cancer in women aged 15 - 44 years (Bruni *et al*., 2016).

Cervical cancer is caused by various factors such as persistence expression of human papilloma virus (HPV) genes, environmental and genetic cofactors. HPV especially types 16 and 18 accounting for about 95 % of reported cases (Lungu *et al*., 1995). The E6 and E7 oncogenes founds primarily in these HPVs, promotes cell transformation by binding

to two important tumour suppressor genes, p53 and pRB respectively, and disrupting their normal cellular functions (Munger *et al*., 1989; Scheffner *et al*., 1990).

In this study, three different human cervical cancer cell lines were studied, which are CaSki, HeLa S3 and SiHa. CaSki is a hypotriploid cell line derived from 40 years old Caucasian female with epidermoid carcinoma of the cervix metastasis to the small bowel mesentery. It contains an integrated HPV type 16 genome (HPV-16, about 600 copies per cell) as well as sequences related to HPV-18. CaSki cells were reported to express beta subunit of human chorionic gonadotropin (hCG) and GbPD type-B tumour associated antigens (Pattillo *et al*., 1977). HeLa S3 is a clonal derivative of the parental cell line HeLa. It is a cervical adenocarcinoma of 31 years old Caucasian female with epithelial-like morphology. HeLa S3 is reported to contain HPV-18 sequences. SiHa is a grade II squamous cell carcinoma from 55 years Japanese female. This cell line was established from fragments of a primary tissue sample with an epithelial-like morphology. It expresses oncogenes like p53 and pRB. SiHa is reported to contain integrated HPV-16 genome with 1- 2 copies per cell.

2.5.9 Prostate cancer

Prostate cancer may originate from basal cells or from differentiated secretory luminal cells of the prostate (Lang *et al*., 2009). It is the second most common cancer mortality with 13 % of all cancer fatalities (Greenlee *et al*., 2001). This cancer fail to show early symptoms, as majority of malignancies develop away from the urethra, at peripheral portion of the gland. Symptoms only arise after local extension and metastases developed. Hesitancy, slowing of the urinary stream, intermittent flow, frequency and urgency to

urinate are few examples of prostate cancer symptoms. Decreased in ejaculating volume and erectile dysfunction also can be noticed as tumour progress.

The PC-3 cancer cell line is an epithelial-like cell line, initiated from a bone metastasis of a grade IV adenocarcinoma derived form a 62 years old Caucasian male. This cells express HLA A1 and A9 genes and can exhibit low acid phosphatase and testosterone-5-alpha reductase activities. PC-3 cells do not express androgen receptors and are therefore androgen-independent (Kaighn *et al*., 1979; Van *et al*., 2003). Another prostate cancer cell line, DU-145 is derived from human prostate adenocarcinoma metastases to the brain of a 69 years Caucasian male and are also androgen independent cells. PC-3 and DU-145 are not hormone-sensitive and do not express prostate-specific antigen (PSA).

2.5.10 Lung cancer

Lung cancer remains the single most common cause of cancer death with nearly 20 % of cancer mortality in 2012 (Ferlay *et al*., 2013). It is a cancer of the respiratory system which can occur anywhere in the airways or lungs. Approximately, 75 % of lung cancer cases are because of smoking tobacco, with an estimation of 85-90 % in U.S. (Furrukh, 2013). Interestingly, women are more likely to be affected than men due to nonsmoking-related lung cancer (Couraud *et al*., 2012). A recent study by International Association for the Study of Lung Cancer showed 51 % of world's lung cancer cases occur in Asia (World Cancer Report 2014) with 21 % of mortality. China is the largest tobacco consumer in the world and smoking death is estimated to be around 2 million in 2030 and are expected to triple by 2050 (Koplan & Eriksen, 2015).

There are three types of lung cancer, majority are non-small cell lung cancer about 85 % with subtypes: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Then, 10-15 % accounts for small cell lung cancers which tend to spread quickly. The least common are lung carcinoid tumours with less than 5 %. It grows slowly and rarely spread. Surgical resection is the most effective option for treatment of lung cancer (Molina *et al*., 2008). However, majority of patients are only diagnosed at an advanced stage. Therefore, chemotherapy and radiation are the most beneficial form of treatment (Pfister *et al*., 2006). But still, treatment at this stage only achieves 16.6 % of 5-years survival rate mainly due to late diagnosis and metastases level (Howlader *et al*., 2013). Apart from tobacco (Shaper *et al*., 2003), lung cancer develops following radiation exposure, asbestos, arsenic contamination (Boffetta, 2004), air pollution (Künzli & Tager, 2005) and alcohol consumption (Freudenheim *et al*., 2005).

The most common cell lines of non-small cell lung cancer used for both basic research and drug discovery are A549 and SK-LU-1. A549 is an epithelial carcinoma derived from a 58 years old male patient. It expresses mutant K-RAS and wild type epidermal growth factor receptor, EGFR. SK-LU-1 is an epithelial adenocarcinoma from 60 years old Caucasian female. It is a slow growing cell line with low-anchorage independent growth capacity in comparison to A549 cell line that is fast growing with high-anchorage independent growth capacity (Goldsmith *et al*., 1991).

2.5.11 Oral cancer

Oral cancer in a malignant neoplasia arises in the oral cavity including lips, tongue, gingiva, mouth floor, parotid and saliva glands. It is a squamous cell carcinoma due to 90 % of the dental area histologically originated in the squamous section. Despite the

progress in research and treatment, it is still remain within top 10 cancer-incident ranking prevalence, being highest in India (Boyle *et al*., 1992; Rivera, 2015). According to Farley *et al*. 2012, about 2.1 % of oral cancer cases are recorded worldwide and fatal cases were around 145,000. American Cancer Society reported oral cancer incidences were increasing in developed countries compared to developing countries.

Major risk factors of oral cancers are tobacco and heavy alcohol consumption. Oral cancer is susceptible to pro-carcinogens in tobacco (Singh *et al*., 2013) while ethanol in alcohol forms a carcinogen compound when oxidized to acetyldehyde (Boccia *et al*., 2008). The synergistic consumption of both alcohol and tobacco increases the occurrence of this cancer. Next, frequent betel-quid (Lee *et al*., 2012) and areca-nut chewing contributes to oral cancer mainly in many parts of Asia. Arecoline in areca nut causes cell death, apoptosis and cell cycle arrest of epithelial cells contributing to the pathogenesis of oral carcinogenesis (Chang *et al*., 2012). Besides that, age and family history (Ankathil *et al*., 1996), HPV infections (Vargas-Ferreira *et al*., 2012), immunosuppressed patient due to other diseases (Epstein, 2007) and exposure to UV light (Czerninski *et al*., 2010) are amongst other factors responsible in inducing oral cancer.

Two different oral cancer cell lines used in this study were ORL-115 and ORL-48. They are surgically resected specimens obtained from untreated primary human oral squamous cell carcinomas of the oral cavity. ORL-48 cell line was developed from an Indian female patient with gum tumour. Besides that, hypermethylation of $p^{16IKK4a}$ and overexpression of MDM2 was observed in this cell line (Hamid *et al*., 2007). In ORL-115, overexpression of EGFR was observed and it was tested positive for HPV types 16 and 3 (Fadlullah *et al*., 2016).

2.5.12 Bladder cancer

Bladder cancer is a malignancy of urinary tract with an incidence being four times higher in men than in woman (Ploeg *et al*, 2009). Most of the bladder cancers are transitional cell carcinoma that arises from the transitional epithelium (Landis *et al*., 1999; Jemal *et al*., 2007). Non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) are the two types of transitional cell carcinomas which are classified histopathologically (Black & Dinney, 2007; Shen *et al*., 2008). Around 70-80 % of bladder cancers represent NMIBC and the remainder, are MIBC with an invasion of the muscular layers of the bladder. Usually patients with NMIBC are successfully treated while most death occurs in patients with MIBC (Black & Dinney, 2007). The less common types of bladder cancer are squamous cell carcinoma (3-5 %), adenocarcinoma (0.5 to 2 %); small cell carcinoma (less than 0.5 %) and sarcoma, carcinosarcoma /sarcomatoid tumours, paraganglioma, melanoma and lymphoma (less than 0.1 %) (Dahm & Gschwend, 2003). Frequent urination and pain during urination are the most common symptoms of bladder cancer (Zeegers *et al*., 2004).

The main risk factor of bladder cancer is cigarette smoking. It has been reported that, smokers have an approximately three fold higher risk of bladder cancer compare to non-smokers due to present of chemicals in the smoke, such as, 2-naphthylamine and 4-aminobiphenyl (Zeegers *et al*., 2000). Besides that, arsenic in drinking water also plays a role as a risk factor. It inhibits sulfhydryl containing enzymes indirectly and interferes with cellular metabolism, such as cytotoxicity, genotoxicity and inhibition of enzymes with antioxidant function (Anetor *et al*., 2007). Risk of bladder cancer also increases following occupational exposure to aromatic amines, such as 2-naphthylamine, 4-aminobiphenyl (Chen *et al*., 2005) and benzidine can be found in the products from the

chemical, dye and rubber industries as well as in hair dyes, paints and fungicides (Yu *et al*., 2002).

Two different bladder cancer cell lines used in this study were RT-112 and EJ-28. Both this cell lines are human bladder carcinomas derived from urinary bladder of Caucasian female. RT-112 is non-invasive while EJ-28 is an invasive cell line. RT-112 was established by Benham *et al*. in 1977 and it expresses the p53 protein.

2.6 Multi drug resistance

A phenomenon, when diseases become tolerant to pharmaceutical treatments, is known as drug resistance. This concept was observed first in bacteria when it became resistance to certain antibiotics. Similar mechanism was also observed in diseases including cancer. Although many type of cancer initially respond to chemotherapy, over the time, they become resistance mostly due to DNA mutation, metabolic changes, drug efflux process and many more causes (Figure 2.7).

Drug resistance often developed due to failure in drug activation at *in vivo* level. Anticancer drugs have to undergo metabolic activation in animal or human, in which the drug interaction with different proteins leads to modification, partial degradation or formation of complex between the drug with other molecules or protein. However, cancer cell can reduce drug activation thus resulting in development of resistance. For example, in the treatment of acute myelogenous leukemia with cytarabine (AraC), the drug will be activated after several phosphorylation events that convert AraC into AraC-triphosphate (Sampath *et al*., 2006). AraC drug resistance develops following mutation in the pathway that causes decrease in AraC activation. Similarly, cytochrome P450 (CYP) system, glutathione-*S*-transferase (GST) superfamily, and uridine diphospho-glucuronosyl

transferase (UGT) superfamily (Michael & Doherty, 2005) are also involved in drug inactivation and activation.

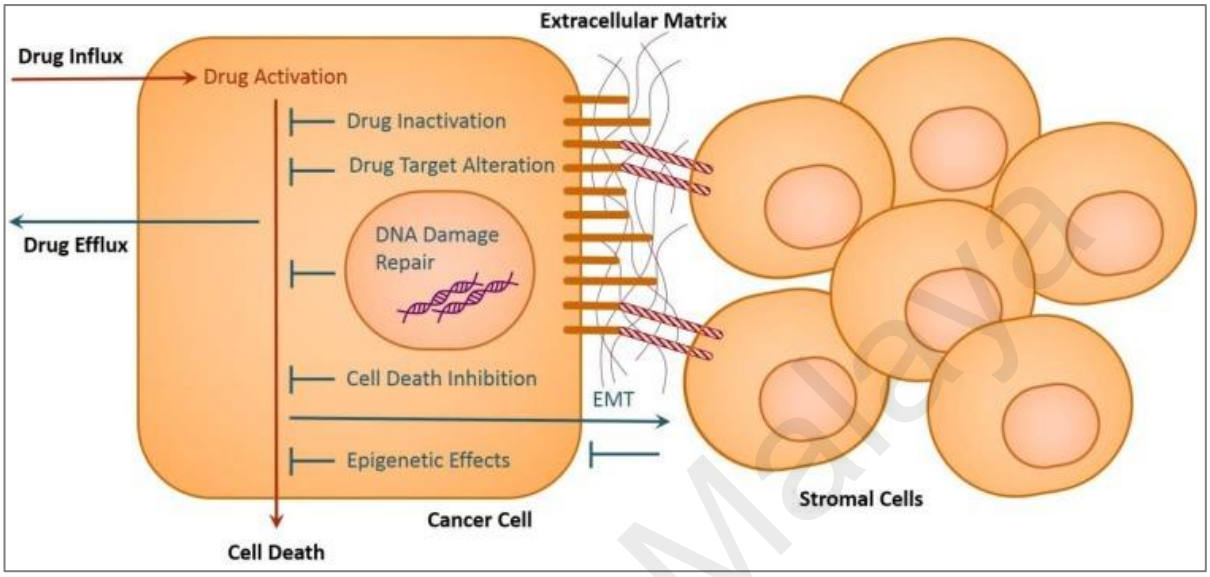


Figure 2.7: Depiction of the primary mechanisms that enable cancer cells to become drug resistant. These include drug inactivation, alteration of drug targets, drug efflux, DNA damage repair, inhibition of cell death, EMT, and epigenetic effects. In the case of EMT, stromal cells assist in this process and signal for improved drug resistance in cancer cells. Cell adhesion molecules on stromal cells and extracellular matrix proteins attach to the cell adhesion molecules on cancer cells. Stromal cells and cancer cells also secrete factors that regulate EMT. The depiction displays a simplified example of these cell interactions. (Adapted with permission from Housman *et al.*, 2014).

Secondly, alteration of molecular targets leads to drug resistance. Certain anticancer drug target, such as, topoisomerase II is an enzyme that prevents DNA from becoming super or under-coiled. The complex between DNA and this enzyme is usually transient, but the drug can stabilize it, leading to DNA damage, inhibition and halting mitotic process. Cancer cell can form resistance via mutation in topoisomerase II gene itself (Hinds *et al.*, 1991; Stavrovskaya, 2000). An important target signaling kinases are of the epidermal growth factor receptor (EGFR) family and their down-stream signaling partners, such as, Ras, Src, Raf, and MEK. Such example is seen in ovarian cancer where mutation in beta-tubulin caused resistance in paclitaxel and other taxanes (Mehta & Fok, 2009).

Next, drug resistance occurs when drug efflux enhanced following drug accumulation. Member of the ATP-binding cassette (ABC) transporter family proteins especially multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP) enable mechanism of efflux and implicated in many drug resistance. They are able to efflux many xenobiotics, including vinca alkaloids, epipodophyllotoxins, anthracyclines, taxanes, and kinase inhibitors, from cells, therefore, protecting cancer cells from first line chemotherapies.

The repair of damaged DNA has a clear role in anticancer drug resistance, when chemotherapy using either directly or indirectly damage DNA. As a result, DNA damage response (DDR) mechanism can reverse drug-induced damage. Therefore, the efficacy of chemotherapeutic drug especially DNA- damaging cytotoxic drugs depends on the failure of the cancer cell's DDR mechanism. If the cancer cell's repair pathway was inhibited or weakened, the drug can able to sensitize cancer cell and enhance efficacy of the therapy. For example, CDDP causes DNA crosslink that leads to apoptosis. However, resistance to CDDP arises due to nucleotide excision repair and homologous recombination, the primary DNA-repair mechanism involved in reversing the platinum damage (Selvakumaran *et al*., 2003; Olaussen *et al*., 2006; Bonanno *et al*., 2014).

Solid tumour turns metastasis via epithelial to mesenchymal transition (EMT) mechanism. Metastasis is a complex process that includes angiogenesis in which tumour cells move outwards to promote tumour spread. Several factors involved in drug resistance during EMT includes, degree of EMT or metastasis grade and signaling process of differentiation which generates more metastasis. For example, the increased expression of integrin αvβ1 in colon cancer positively regulates transforming growth factor β (TGFβ)

expression, which is required for EMT, and it further serves as a survival signal for cancer cells against drugs (Bates & Mercurio, 2005).

The heterogeneity in cancer cells leads to drug resistance. Studies has shown, a fraction of cells within this heterogeneity population have stem cell properties and are usually drug resistance. During treatment, drug usually kills cells those are susceptible to it while the resistant cells survive, expand and contribute to pathology over time (Parkin *et al*., 2013).

Another factor involved in cancer cell resistance is cell death inhibition through either apoptosis or autophagy. Increased proliferation can be seen with cell death inhibition. Drug resistance cancer cell can be effectively treated by using drug combination. One drug makes the cancer cells susceptible to death while the other cytotoxic drug kills them in their vulnerable state. TRAIL induces apoptosis through JNK activation (Sarkar & Faller, 2011; 2013), and inhibition of the JNK signaling pathway leads to a decrease in cisplatin-induced apoptosis.

2.6.1 Combinatorial chemotherapy

Implication of drug resistance, tumour cell heterogeneity and successful clinical trial leads to increased attention on usage of combination chemotherapy as a best strategy to treat cancer. Drug combination can be formulated with two, three or even four different drugs to treat one disease. When drugs are combined together their interaction can be categorized as additive, synergistic or antagonistic. In additive interaction, the effect of two drugs together is merely the sum of the effect of each drug; synergistic interaction, when the effect of two drugs are greater than the sum of the individual effect while in

antagonistic interaction, effect of two drugs are less than the sum of their individual effects. Synergistic combination of two or more drugs gets attention in cancer treatment since it can overcome toxicity and other side effects associated with increased doses of individual drug by giving greater effect on cancer cell clearance.

Combination chemotherapy treatment offers several benefits. Applying multiple target drugs can increase genetic barriers and delay cancer adaptation process while multiple drug targets with similar pathway can interact synergistically to give higher therapeutic efficacy and target selectivity (Lehar *et al.*, 2009; Wang *et al.*, 2011; Liu *et al.*, 2013). Moreover, the advantages of combinations include the ability to replace current expensive anti-cancer therapies through the use of cheaper drug cocktails (Kashif *et al.*, 2015). For example, natural products such as curcumin is shown to enhance effects of the chemotherapeutic drug, gemcitabine by sensitizing human bladder cancer cell and induces apoptotic effects through NF-κB inactivation (Kamat *et al.*, 2007). Similarly, 1'S-1'-acetoxyeugenol acetate (AEA) a phenylpropanoid in combination with paclitaxel chemosensitizes human breast cancer cells and enhances its apoptotic effects (In *et al.*, 2011).

2.7 Cancer and inflammation

The functional relationship between inflammation and cancer is not new. In 1863, Virchow hypothesized that the sites of chronic inflammation are the origin of cell proliferation which lead to cancer formation (Virchow, 1863). During tissue injury which proceeds to wounding, cell proliferation is enhanced while tissue regenerates. Proliferation and inflammation decreases after repair is completed. In contrast, when inflammation is unregulated, it can become chronic including malignant cell

transformation in the surrounding tissue. Both inflammatory responses and carcinogenic process shares various molecular targets and signaling pathways, such as, apoptosis, increased proliferation rate and angiogenesis. The risk of cancer is increased with elevated inflammation. For example, chronic gastritis by *Helicobacter pylori* increases chances of gastric cancer (Yoshida *et al*., 2014), inflammation followed by exposure of asbestos, infections, smoking, and silica causes lung cancer (Vainio & Boffetta, 1994), *E. coli* infection of prostate induces prostate cancer (Krieger *et al*., 2000) and gall bladder stone-associated chronic colic cystitis induces gall bladder carcinoma (Fox *et al*., 1998; Levin, 1999). During chronic inflammation, cells that infiltrating the tumour microenvironment releases bioactive molecules like cytokines, growth factors and chemokines which altogether establish an inflammatory microenvironment (Coussens & Werb, 2002). Inflammation at the molecular level is determined through the expression of a number of inflammatory transcription factors, such as, NF-κB and signal transducers and activators of transcription 3 (STAT3). They are two of the most important transcription factors in inflammatory pathways that play major roles in tumourigenesis and thus can be considered targets for prevention and therapy of cancer (Aggarwal *et al*., 2006; Lin & Karin, 2007). Inflammation also influences the host immune response to tumour, thus can be used in cancer immunotherapy. Also inflammatory enzymes, such as, COX-2, MMP-9, 5-LOX and phospholipase PLA_2 and inflammatory cytokines such as TNF, IL-1, IL-6, IL-8 and chemokines are important mediators (Aggarwal *et al*., 2006).

2.8 Animal model studies

Cell culture and animal experiments are essential to determine efficacy of pharmacodynamics and fundamental mechanism of novel anti-cancer drugs. Tumours are made of heterogeneous population of cells with increased genetic modification that interact with host environment, leading to activation of inflammatory and immune

systems. Besides, tumour cells not only interact with each other and the extracellular matrix but also with the host cells including endothelial cells, fibroblast, immune cells and inflammatory cells. Screening a novel drug in cell line system aims to identify growth rate, cytotoxicity and mechanism of action of the drugs (Shoemaker, 2016). However, in this two-dimensional cell system, culture conditions alone do not approximate the complexity of tumours in patient. The cells are highly selected where they fail to create heterogeneity in genetic and phenotypic of tumour cells as in human. Furthermore, additional genetic and epigenetic changes occur due to propagation of cells in cell culture (Houshdaran *et al*., 2010; Hennessey *et al*., 2011). Therefore, validation of cell culture results in animal model would give a clear picture on the drug efficiency prior to clinical trials. *In vivo* animal test is aimed to show safety and to verify the tested drug has scientific merit for further development. Importantly, Food and Drug Administration (FDA) required animal model test to be continued prior to administration of the new molecular entity in humans (Junod, 2013). Therefore it is essential to validate efficiency of drug in a most suitable system which mimics human tumours closely. For this reason, animal model has gained vast acceptance for a wide variety of diagnostic and therapeutic applications.

In cancer research, mouse cancer models are frequently used to validate *in vitro* results. The most commonly used rodent models are xenograft and chemically or genetically induced mouse models.

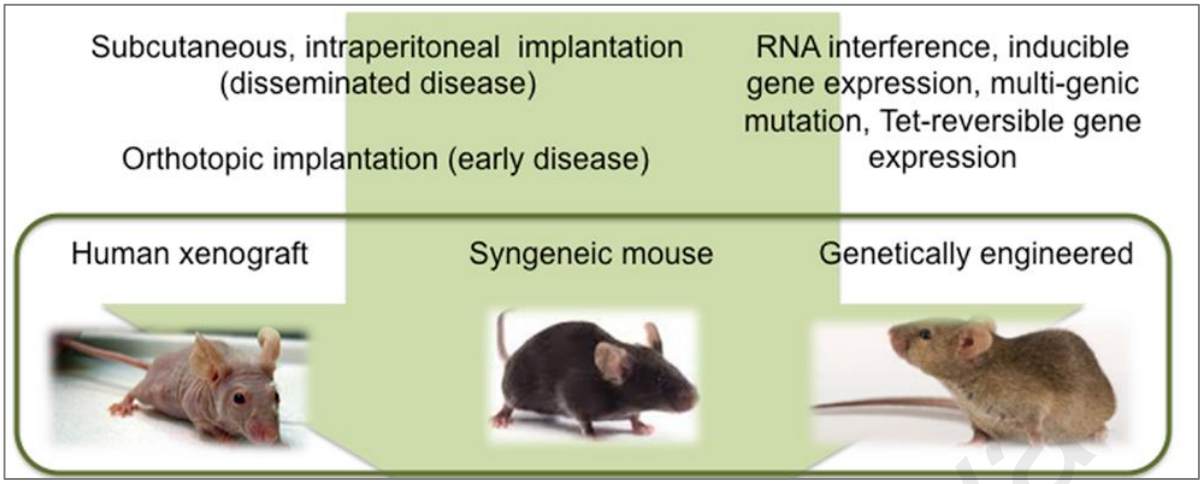


Figure 2.8: Technological advances in mouse models. (Adapted with permission from House *et al.*, 2014).

2.8.1 Xenograft models

Xenograft cancer model are generated using compromised rodents when human or animal cancer cells are transplanted either ectopic, under the skin or orthotopic, into the organ of tumour origin (Bibby, 2004; Ruggeri *et al.*, 2014). The most common type of immunocompromised mice used in cancer research are nude mice (*Foxn1*^{nu}), severe combined immunodeficiency (*scid*) mice (*Prkdc*^{scid}), RAG1 and RAG2-deficient mice, and NOD-*scid* and NOD-*Rag1*$^{-/-}$ mice (Shultz *et al.*, 2007). The athymic nude mice has mutation of *Forkhead box protein N1* (*Foxn1*^{nu}) gene which lead to compromised immune system. Since these mice are without thymus, they have an incomplete T cell development. However only about 20-40 % of successful tumour cell line generation is seen as these mice still have a high NK cell activity (HogenEsch & Nikitin, 2012). On the other hand, SCID mice carry a mutation in DNA-dependent protein kinase complex thus, leads to absence of T and B lymphocytes resulting in complete failure of their immune system (Shultz *et al*., 2007). The advantages of using xenograft models are inexpensive and ease of *in vivo* tumour generation from human and animal cancer cell lines. However, its major drawbacks are when this model fails to completely represent behavior of naturally occurring cancers in human (Sharpless & Depinho, 2006; Frese & Tuveson,

2007). Apart from that, lack of stroma-tumour interaction and superficial vascularization of xenograft tumour limits the rapid translation of research to the human. Different degrees of immune-deficiency in mice also vary the acceptance rate of xenograft as well as resulting in different biological behaviors and response to drug. Tumours implanted in *scid* and *Rag2*$^{-/-}$ mice had a different response to cancer treatments compared with tumours implanted subcutaneously in nude mice (Yoshimura *et al*., 1997). Despite of this weakness, xenograft models are still used frequently and reported to have a great predictive value for selecting prospect anticancer agent for clinical and human trials. For example, comparison of drug efficacy in patients and their xenografts established from biopsies revealed that patient-derived xenografts are highly predictive for responsiveness (90 %, 19/21) and resistance (97 %, 57/59) (Dong *et al*., 2010).

2.8.2 Genetically engineered animal models

Following incapability of xenograft models to create real tumour microenvironment as in human, genetically engineered animal model is developed to recapitulate the genetics and histology of human tumour. Genetically engineered animals are organisms in which specific genes have been altered either added or deleted to create suitable model for animal or human disease, in this case for cancer research. In this mice model, oncogenes are activated and tumour-suppressor genes are inactivated somatically, through temporally controlled and tissue-specific expression of CRE recombinase (tyrosine recombinase enzyme derived from the P1 bacteriophage). These animals with developed tumour at specific tissue will be treated with the anticancer drug and serially assessed for responses (Sharpless & Depinho, 2006). There are several benefits of this model, such as, it could provide good preclinical screening and safety testing for use in identification and optimization. Besides that, genetically engineered mice can provide information on gene

function which is relevant for human health and disease (Vandamme, 2014). Importantly, formation of tumour, its microenvironment and interaction with surrounding cell system closely resembles those in human to the extent of disease progression including formation of metastasis (Heyer *et al*., 2010; Cheon & Orsulic, 2011). For example, genetically engineered mice with inactivation of tumour suppressor gene *p53* and *Rb* has resulted in development of high grade serous adenocarcinoma metastasis which closely similar to human (Donehower *et al*., 1992).

2.8.3 Syngeneic models

Syngeneic tumour models are where a mouse tumour growing in mice of the same strain in which the tumour originated. For example, mice origin breast cancer cell line 4T1, ortho-grafted in mammary fat pad of BALB/c mice to generate breast tumour (Corpet & Tache, 2002). Advantages of this mice model is they are inexpensive, comes in wide variety of tumour type (such as MBT-2 in bladder cancer; 4T1 and EMT-6 in breast cancer; CT26 and MC38 in colon cancer; Pan02 in pancreatic), tumour grow very fast and most importantly cancer cells grow in immunocompetent hosts without any rejection. However, the limitation is the tumour cells are rodent origin, thus express the mouse or rat homologous of the desired targets (Teicher, 2006). Some examples of syngeneic models include: 4T1 metastatic breast cancer in immunocompetent BALB/c mice; Renca metastatic renal cell carcinoma (RCC) that is modeled in BALB/c mice (Tracz *et al.*, 2014); Lewis lung cancer (LLC) in immunocompetent C57BL/6 mice (Perez-Soler, 2004).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Dulbecco modified Eagle medium (DMEM) supplemented with 4.5 g glucose/L and 300 mg/L L -glutamine was purchased from Hyclone Laboratories Inc, Logan, Utah. Roswell Park Memorial Institute 1640 (RPMI-1640) was purchased from Thermo Scientific Hyclone, USA. Fetal bovine serum (FBS) was purchased from Lonza Inc. (Allendale, New Jersey, USA). Keratinocyte serum-free medium (KSFM) and TRIzol reagent were obtained from Invitrogen, Grand Island, New York. Minimum essential medium alpha (MEM-α) was purchased from Life Technologies, USA. Cisplatin and 3-(4, 5-dimethylthiazol-2-gl)-2, 5-diphenyl-tetrazoliumbromide (MTT) reagents were obtained from EMD Chemicals Inc. Middlebrook 7H10 agar and Middlebrook 7H9 broth were obtained from Sigma-Aldrich, Germany. *Mycobacterium indicus pranii* was provided by Prof. Dr. Niyaz Ahmed, Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad, India.

3.2 Cultivation of cancer cells

A total of fifteen human cancer cell lines were used in this study: breast adenocarcinoma (MCF-7 and MDA-MB-231), hepatocyte carcinoma (HepG2), cervical (CaSki and HeLa S3), prostate carcinoma (PC-3 and DU-145), lung adenocarcinoma (A549 and SK-LU-1), oral (ORL-48, ORL-115 and ORL-136) and bladder (RT-112 and EJ-28). Immortalized human cell lines from nasopharyngeal epithelial (NP-69), breast epithelial (MCF-10A) and keratinocyte (HaCaT) were used as representatives of non-cancerous cells. 4T1 mouse mammary cell line was used in *in vivo* animal study. All cell lines were obtained from ATCC except human oral cancer cell lines which were obtained from Cancer Research Initiative Foundation (CARIF, Malaysia) and bladder cancer cell lines

were obtained from Prof. Dr. Abhimanyu Veerakumarasivam, Perdana University, Malaysia. HaCaT, HepG2, CaSki and HeLa S3 were cultured in DMEM, while MCF-7, PC-3, MDA-MB-231, A549, RT-112, EJ-28 and DU-145 cells were cultured in RPMI 1640, supplemented with 10.0 % (v/v) FBS. SK-LU-1 cells were cultured in MEM-α supplemented with 10 % (v/v) heat inactivated FBS. Human oral cancer cell lines were cultured in DMEF and supplemented with 10.0 % (v/v) FBS. The cell lines used in this study were listed in Table 3.1.

All cells were grown as monolayers and were maintained in a humidified CO_2 incubator at 37 °C in 5.0 % CO_2 and 95.0 % air and were split when 80-90 % confluency was achieved on the culture flask surface. Cells were washed with 1x PBS before 2.0 ml of 0.25 % (v/v) trypsin solution added for 5 min at 37 ^{o}C to allow the detachment of cells from culture flask. Then, 6 ml of growth media was added to inactivate trypsin activity and further pipetted into a 15.0 ml falcon tube. Trypsinised cells were centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. The cell pellets were re-suspended in 8 ml of fresh growth media and split into prepared culture flasks for further usage.

Table 3.1: List of different types of cancer and normal cell lines used in this study, accompanied by the indication of sources and various culture media used for cultivation

Cancer type	Cancer cell	Source	Culture media
Human			
Breast	MCF-7	ATCC	RPMI 1640
	MDA-MB-231	ATCC	RPMI 1640
Cervical	CaSki	ATCC	DMEM
	SiHa	ATCC	DMEM
	HeLa S3	ATCC	DMEM
Lung	A549	ATCC	RPMI 1640
	SK-LU-1	ATCC	MEM-α
Prostate	PC-3	ATCC	RPMI 1640
	DU-145	ATCC	RPMI 1640
Liver	HepG2	ATCC	DMEM
Bladder	EJ-28	University Perdana	RPMI 1640
	RT-112	University Perdana	RPMI 1640
Oral	ORL-48	CARIF	DMEF
	ORL-115	CARIF	DMEF
Mouse			
Breast	4T1	ATCC	RPMI 1640
Normal cell			
Immortal Keratinocyte	HaCaT	ATCC	DMEM
Breast Epithelial	MCF-10A	ATCC	MEBM
Human nasopharyngeal epithelial	NP-69	ATCC	KSFM

3.2.1 Preparation of frozen stocks

Confluent cells were washed with 1X PBS and detached from flask surface with 2.0 ml of 0.25 % (v/v) trypsin solution for 5 min at 37 ºC. Equal volume of media supplemented with 10.0 % (v/v) FBS was added to inactivate trypsin activity. Cell suspension was then pipetted into 15.0 ml tubes and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the cell pellet was suspended in fresh media containing 20 % FBS and 10 % DMSO as the cryo-protecting agent. Pen-Strep, 1 % (v/v) was added to this mixture to prevent bacterial contamination due to their effective combined action against gram-positive and gram-negative bacteria. Several stocks of 1.0 ml aliquots were prepared in 2.0 ml cryovials, frozen gradually at -20 ºC for 3 hours followed by in -4 ºC for 3 hours. Finally the stocks are stored in liquid nitrogen at -196 ºC for long term storage.

3.2.2 Thawing of cryopreserved cells

Cryopreserved cells were removed from liquid nitrogen and thawed immediately in 37 ºC for 2 min in a water bath. Thawed cell suspension was then diluted in 5 ml of growth medium containing 10.0 % FBS and centrifuged at 1500 rpm for 5 min to remove cryo-protective agent DMSO. The pellet was re-suspended in fresh medium containing 10.0 % FBS and were split into T25 cm^2 or T75 cm^2 flasks and incubated at 37 ºC in a 5.0 % CO_2 and 95 % humidity level atmosphere.

3.2.3 Cell counting

Haemacytometer was used to determine number of cells present in a specific population via dye exclusion viability assay. Monolayer of cells were detached from flask by trypsinization, centrifuged and re-suspended in media. Approximately 20.0 µl of cell

suspension was mixed with equal amount of 0.08 % trypan blue, (Merck, Germany) dye solution. A volume of 10.0 µl of the mixture was transferred to a haemacytometer counting chamber and spread evenly by capillary action. Using an inverted microscope at 100X magnification, the number of unstained viable cells in each of the quadrant was counted and the average number of cells was obtained. Each quadrant represents a 0.1 mm^3 or 10^{-4} ml volume and the concentration of cells were determined as shown below with a dilution factor of 2. After viable cells were counted, the haemacytometer slides and glass cover slip was immediately rinsed and cleaned with 70 % (v/v):

$$\text{Cell concentration (cells/ml)} = \frac{\text{Average number of cells counted}}{\text{Volume counted, ml}} \times \text{Dilution Factor (2)}$$

3.3 Bacterial cultures

MIP was cultured in Middlebrook (MB) 7H9 broth supplemented with 5 ml glycerol, 0.2 % Tween-80, 10 % albumin-dextrose complex enrichment (ADC) and incubated at 37 ^{o}C, 100 rpm agitation. The number of viable mycobacterial cells in each single-cell suspension was determined by standard plate counting. Briefly, turbidity of the broth was measured by taking absorbance readings at 600 nm. For each time point, serial dilution of the cell suspension was prepared. One hundred microliters from each dilution was plated onto each of three 7H10 agar plates supplemented with 10 % albumin-dextrose complex enrichment (ADC) and incubated at 37 ^{o}C. Colony counts (CFU) were determined after the incubation of plates after 10 days using given formula:

$$\text{Number of bacteria} = \frac{\text{Number of CFU (single colony)}}{\text{Volume plated (0.1 ml) x dilution factor}} = \text{CFU/ml}$$

A standard curve was plotted comparing the OD 600nm of the broth culture and with the number of viable cells/ml, the regression equation obtained was used to standardize number of bacteria used for treatment.

3.3.1 Preparation of MIP fractions

MIP fractions: live bacteria (LB), live supernatant (LS), heat killed bacteria (HKB) and heat killed supernatant (HKS) were harvested in the log growth phase by centrifugation at 3400 rpm for 15 min. LB suspension in PBS contains 6×10^9 MIP cells/ml was suspended in 0.9 % w/v sodium chloride and 0.01 % (w/v) thimerosal. HKB and HKS were prepared by inactivating live bacteria and live supernatant at 60 °C for 20 min. The various cancer cells were treated with different volumes (10–100 µl/ml/10^6cells) of MIP fractions.

3.3.2 Identification of optimum temperature in preparation of heat killed bacteria

Live bacteria fraction was harvested in log growth phase and heat inactivated at 20 min in a series of temperature at 30 °C, 40 °C, 50 °C, 60 °C and 70 °C. The bacteria were then cultured on Middlebrook (MB) 7H10 agar plate supplemented with 5 ml glycerol, 0.2 % Tween-80, 10 % albumin-dextrose complex enrichment (ADC) and incubated at 37 ^{o}C with agitation of 100 rpm.

3.4 Preparation of ACA

Purified ACA with molecular weight 234.4 g was dissolved in an organic solvent, DMSO to make both stock and working solutions. To make a 20X ACA stock solution with final concentration 20.0 mM, 46.9 mg of pure ACA was dissolved in 10.0 ml of DMSO

(Merck, Germany). The solution was then vortexed vigorously to ensure that ACA had completely dissolved in the DMSO. Then 2X dilution on the ACA stock solution was made to obtain 1X ACA working solution with a final concentration of 10.0 mM. The ACA solutions were kept in 4 °C. Before treatment in cell culture, the ACA working solution was diluted with culture medium to a final DMSO final concentration of less than 0.05 %, at which no solvent-induced cytotoxic effects of DMSO *per se* was observed.

3.5 Preparation of cisplatin

A cisplatin solution (Sigma Aldrich, Germany) was prepared freshly before use at 3.3 mM in PBS and diluted to a 0-150 µM concentrations.

3.6 Agar diffusion assay

Agar diffusion assay was performed to identify bacterial inhibition by ACA and CDDP. MIP at a density ~6.0×10^9 MIP cells/ml at 1.5 OD 600, were spread onto Middlebrook 7H10 agar plated plates. Sterile filter discs (~0.6 cm) soaked in either ACA, CDDP or neomycin was placed on the middle area of the agar surface. A total amount of 10 µl aliquot of ACA (3.0, 10.0, and 20.0 mM), CDDP (30, 100 and 200 mM) and neomycin (0.5, 1.0, 3.0, 5.0, 8.0 and 10.0 µg/ml) were loaded onto the disks and then incubated at 37 ^{o}C for 4 days before analysis.

3.7 Cytotoxicity assay

3.7.1 Preparation of MTT solution

MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent with a final concentration 5.0 mg/ml was prepared by adding 60.0 mg of MTT (Calbiochem, USA) to 12.0 ml of 1x PBS. The reagent was vigorously shaken and vortexed to ensure that the MTT granules were completely dissolved in PBS and stored at 4 °C in the dark. MTT working solutions were stored in dark at room temperature before usage.

3.7.2 MTT cell viability assay

MTT assay was carried out to measure cytotoxic effects of MIP fractions, ACA, CDDP as standalone and in combination on various cancer cell lines by measuring MTT dye uptake and metabolism. MTT is a substrate which is reduced by dehydrogenase enzymes present in the mitochondria of viable cells. In MTT assay, the intensity of the purple formazan product were measured and used to quantify viable cells in culture. A total of 1.0 x 10^4 cells were plated in triplicates at 100 µl /well in a 96-flat bottom well plate and incubated overnight at 37 °C to allow adherence to the well surface. ACA, CDDP and various MIP fractions were treated at various concentrations (ACA: 0-50 µM; MIP fractions: 10–100 µl/ml/10^6cells; CDDP: 0-150 µM) then incubated for 24 hr. A total volume of 20 µl of MTT reagent (5.0 mg/mL) was added to each well. The plate was left on a shaker for 10 min and incubated in the dark at 37 °C. After 1 hour of incubation, the spent medium containing excess dye was aspirated and 200 µL of DMSO was added to dissolve the purple formazan precipitates. Results were obtained using micro-titer plate reader (Tecan Sunrise, Switzerland), to detect absorbance at a test wavelength of 570 nm, and a reference wavelength of 650 nm. IC_{50} values were determined according to the

following equation: % cytotoxicity = [(absorbance value of solvent- absorbance value of MIP)/ absorbance value of untreated cells] x 100 %.

3.8 DNA fragmentation assay

Conformation of apoptosis-mediated cell death induced by MIP HKB was carried out using the ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen, USA). Cancer cells were grown on 6-well plates at concentration of $3x10^5$ cells/well for 24 hr followed by treatment at IC_{50} concentration for 6, 12 and 24 hr. Both detached and attached cells were collected and centrifuged for 5 min at 500 g. Supernatant was discarded and the cells were lysed with 35 µl TE lysis buffer by pipetting up and down several times. Then, 5 µl of enzyme A was added to the crude lysate. The mixture was gently vortexed and incubated at 37 °C in water bath for 10 min. A volume of 5 µl enzyme B solution was added to each samples and incubated at 50 °C for 30 min in water bath until the lysate became clear. Next, 5 µl of ammonium acetate solution and 100 µl of absolute ethanol (kept at -20 °C) were added to each sample. The mixture was vortexed and DNA was allowed to precipitate at -20 °C, 10-15 min. Samples were centrifuged for 10 min at 12000-14000 rpm to collect the precipitated DNA. The supernatant was carefully discarded. The precipitated DNA was washed with 0.5 ml 70 % cold ethanol followed by 10 min centrifugation at 12000 to 14000 rpm. Supernatant was discarded and DNA pellet was allowed to air dry for 10 min at room temperature. A 30 µl of DNA suspension buffer was added and re-suspended by carefully pipetting up and down several times. The DNA suspension was stored at -20 °C for further usage. Extracted DNA was analyzed on a 1.0 % (w/v) agarose gel electrophoresis and stained with ethidium bromide. Fragmentation of DNA was observed under UV illumination and visualized using a gel documentation system (Alpha Inotech, USA).

3.8.1 DNA quantification

DNA quantification was carried out using nanodrop. Exactly, 1 µl sample was added into 9 µl of distilled water in microcentrifuge tube which gives a 10X dilution. The mixture was vortexed and centrifuged prior to quantification. A total of 1 µl mixture was added into nanodrop and DNA was quantified. Distilled water was used as a blank control. DNA quantification was performed based on absorbance values at 260 nm wavelength. Absorbance ratio for $A_{260/280}$ and $A_{260/230}$ were obtained to assess the purity of DNA samples against protein and solvent contamination, respectively.

3.8.2 Agarose gel electrophoresis

Visualization of apoptotic total DNA from cells treated with MIP was done using agarose gel electrophoresis. Firstly, 1.5 % of agarose gel was prepared by adding 1.5 g of agarose, low EED (Amresco, USA) to 100 ml of 1X TBE buffer (Amresco, USA). The mixture was then heated for 2 min using a microwave oven (Panasonic, Malaysia) until the powder was melted. The mixture was then cooled for 30 sec under running tap water before GelStain (TransBionovo, China) is added before the mixture was poured into a gel casting tray with an attached comb. This mixture was allowed to solidify for approximately 30 min at room temperature. Then the comb was removed and gel was submerged in 1X TBE buffer inside an electrophoresis chamber.

A total of 10 µl of DNA sample was mixed with 2.0 µl of 6X loading dye before they were loaded into the wells. DNA molecular weight marker used was 10 µl of 1000 bp DNA ladder (Calbiochem, USA). Agarose gel was electrophoresed for 1 hr, 120 V at 80 mA. When the electrophoresis was completed, the gel was viewed and photographed under UV using a gel documentation system (Alpha Inotech, USA).

3.9 Protein expression analysis

3.9.1 Extraction of cytoplasmic and nuclear fractions

Nuclear and cytoplasmic proteins were extracted from human tumour cell line treated with MIP alone or in double (MIP/ACA and MIP/CDDP) and triple (MIP/ACA/CDDP) combinations. For NF-κB protein analysis, cells were treated with TNF-α (20 ng/ml) for 1 hour. Following treatment, the supernatant with detached cells were washed with PBS. The remaining cells were then detached with 0.25 % trypsin (Lonza, USA)-EDTA (Gibso, USA) solution and centrifuged at 1500 rpm for 5 min. Then, the supernatant was discarded and collected cells were washed with 1xPBS to completely remove remaining media. Extraction of both the nuclear and cytoplasmic proteins from whole cell lysates was performed using the NE-PER® Nuclear and Cytoplasmic Extraction Kit (Pierce, USA) according to the manufacturer's protocol. Briefly, the cell pellet was re-suspended in 100.0 µl ice-cold CER I containing a cocktail of 1X protease and phosphatase inhibitors (Pierce, USA), and vortexed vigorously. Cell lysates were incubated on ice for 10 min to allow complete cell lysis. Then, 5.5 µl of ice-cold CER II was added to the mixture and vortexed for 5 sec on the highest setting and re-incubate on ice for 1 min. Lysates were then vortexed for 5 sec on the highest setting before being centrifuged for 5 min at 4 °C at the maximum speed of -16,000xg using refrigerated centrifuge to collect cytoplasmic fraction (supernatant). The pellet containing intact nuclei was re-suspended in 50.0 µl of ice-sold NER containing 1x protease and phosphatase inhibitors followed by vortexing at the highest setting for 15 sec every 10 min, for a total duration of 40 min. Then, samples were centrifuged at 16,000xg for 10 min at 4 °C to collect the supernatant that contains nuclear fraction to a clean pre-chilled tube.

3.9.2 Protein quantification

Protein concentration was determined using Quick Start Protein Assay kit 2 (Bio-rad, USA). Reagent A and B were kept at room temperature. A total of 1 µl sample with 9 µl distilled water (10X dilution) was prepared. Both reagent A and reagent B (200:40) were added into each sample. Then, 10 µl of distilled water were used as a loading control. Samples were vortexed to allow proper mixing and centrifuged prior to 37 °C incubation for 30 min. Then, the mixture was allowed to cool down at room temperature for 10 min. Next, 1 µl of mixture were analyzed using nanodrop to identify protein concentration in µg/ml.

3.9.3 Protein normalization

For PARP protein detection of both the cytoplasmic and nuclear protein concentrations were normalized with distilled water to a final concentration of 3.0 ng/ml and 2.0 ng/ml respectively, before proceeding with SDS-PAGE fractionation. Normalized cytoplasmic and nuclear fractions were mixed in a ratio of 1:2 to enhance the detection of PARP activation. For other protein detections (NF-κB and intrinsic apoptotic proteins) of both the cytoplasmic and nuclear protein concentrations were maintained to a final concentration of 1.0 mg/ml. A final volume of 20.0 µl protein sample was mixed with 4.0 µl of 5X lane marker reducing sample buffer (Thermofisher, USA).

3.9.4 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared to fractionate extracted cytoplasmic and nuclear proteins from treated and untreated cancer cells according to protein size. Two different concentrations of acrylamide gels were used, 12 % for separating proteins ranging in size between 14-70 kDa and 7.5 % to

separate protein size between 24-205 kDa as shown in Table 3.2. Two mini gels (18.0 cm x 16.0 cm x 0.75 cm) were prepared each time by clipping four glass plates (Bio- Rad, USA) together on the casting tray (Bio-Rad, USA). All reagents were mixed in the order as listed in Table 3.2 with freshly prepared ammonium persulfate, APS being added last to start the cross linking reaction. The resolving gel was loaded until 75 % of the glass plate was filled and allowed to polymerize for 45 mins. A layer of 0.1 % (v/v) SDS (Promega, USA) was added during the polymerization to prevent oxidization and dehydration of resolving gel. Once solidified, the 0.1 % SDS solution was removed using Kim-Wipe (Kimberly-Clark, Canada) and the 4.0 % (w/v) stacking gel was overlaid on the separating gel and 10-well gel comb with a 0.75 mm thickness was inserted to prepare the wells. Upon polymerization, the gels were transferred to a Mini-PROTEIN® 3 cell gel tank (Bio-Rad, USA) and the gel combs were gently removed. Then, 1X TGS running buffer was then poured into the tank until the gel was submerged (~900 ml). A total of 20.0 µl of denatured protein and 5.0 µl of Biotinylated protein ladder (Cell signaling) were loaded into each gel. Gel electrophoresis was performed by running the gel at 110 V, 400 mA for 20 min using Power Supply-Power Pac (Bio-Rad, USA) to allow the samples to align before entering the resolving gel, followed by 150 V, 400 mA, 45 min to resolve the protein samples.

Table 3.2: List of reagents used for the preparation of 4 % stacking gel, 7.5 % and 12 % of resolving gel for SDS-PAGE

1.0 mm thickness gel	**Stacking gel**	**Resolving gel**	
	4 %	**7.5 %** (24-205 kDa)	**12 %** (14-70 kDa)
40 % Acrylamide	500 µl	2.82 ml	4.5 ml
0.5 M Tris-HCl (pH 6.8)	1.26 ml	-	-
1.5 M Tris-HCl (pH 8.8)	-	3.75 ml	3.75 ml
10 % SDS	50 µl	150 µl	150 µl
Distilled water	3.18 ml	8.2 ml	6.52 ml
TEMED	5 µl	7.5 µl	7.5 µl
10 % Ammonium persulfate (APS)-> 10 mg in 100 µl RNase free water, only add it when ready to load	25 µl	75 µl	75 µl
Bromophenol blue	10 µl	-	-
Total volume	5 ml	15 ml	15 ml

3.9.5 Western blotting

Upon completion of electrophoresis, the layer of stacking gel was removed from resolving gel using COMB. Proteins separated in resolving gel were transferred onto 0.2 µm nitrocellulose membrane using 1x Tris-Glycine-SDS (TGS) transfer buffer with 20.0 % methanol (Merck, Germany). Prior to transferring, the nitrocellulose membrane and thick blotting papers were pre-equilibrated in the transferring buffer for 5 min.

A transfer sandwich was made of, first blotting paper, nitrocellulose membrane, SDS gel and finally second blotting paper and placed in a TransBlotter-SD Semi Dry Transfer Cell (BioRad, USA). A blotting roller was used to remove the presence of air bubbles between each layer of the transfer sandwich. Then, proteins in the gels were transferred onto nitrocellulose membrane at a constant 110 V, 200 mA, 5.0 watt for 90 min using MP-

2AP Power Supply (Major Science, Taiwan). To determine the efficiency of the electrophoretic transfer, the transferred proteins on the membrane were stained with 0.1 % (w/v) Ponceau S (Sigma, USA) in 5.0 % acetic acid for few minutes until bands were visible.

Upon checking the efficiency of transferred proteins, the membrane was then de-stained twice with distilled water for 5 min each while shaking. After washing was completed, membranes were blocked for 1 hr while shaking at room temperature in a blocking buffer consisting of 1 % BSA (Calbiochem, USA) or skim milk, 1X TBS buffer, 0.05 % v/v Tween-20 (Promega, USA) to prevent non-specific background binding of the primary and secondary antibodies. Blocked membranes were incubated while shaking in primary antibody (Table 3.3) in 10.0 ml of blocking buffer at room temperature for 30 min followed by overnight incubation at 4 °C. Then, membranes were washed three times with 1x TBST for 5 min while shaking at room temperature. Membranes were incubated in secondary antibody together with biotin in 10.0 ml blocking buffer for 1 hr while shaking at room temperature. Membranes were washed 3x with TBST for 5 min each followed by single wash in 1X TBS for 5 min. Membranes were treated with 1 ml of highly sensitive chemiluminescent detection reagent, ECL to enhance protein detection.

Table 3.3: List of primary antibodies

Antigen of Primary antibody	Isotype	Brand	Dilution	Size of band
GAPDH	Rabbit IgG	Cell signaling	1:100	37 kDa
NF-κB p65	Rabbit IgG	Cell signaling	1:1000	65 kDa
IκBα	Rabbit IgG	Cell signaling	1:1000	39 kDa
Apaf-1	Rabbit IgG	Cell signaling	1:1000	135 kDa
Caspase-9	Mouse IgG	Cell signaling	1:1000	47, 37, 35 kDa
PARP	Rabbit IgG	Cell signaling	1:1000	116, 89 kDa

3.10 *In vitro* combination therapy

MTT assay was performed on various human cancer cells in order to evaluate *in vitro* double and triple combination effects of MIP/ACA, MIP/CDDP and MIP/ACA/CDDP. A total of 20,000 cells of each cancer cell lines were plated in triplicates at 100.0 µl/well in a 96-flat bottom well plates and incubated for 24 hr at 37 °C to allow cell adherence to the well surface. After 24 hr incubation, cells were treated with MIP/ACA, MIP/CDDP or MIP/ACA/CDDP in combination at various concentrations at 1:1:1 ratio. Wells containing only media were used as negative control. Serial dilution of cells were carried out from $2x10^4$ cells/well, $1x10^4$ cells/well, $5.0x10^3$ cells/well and $2.5x10^3$ cells/well to construct standard curve via quantification of absorbance. After 24 hr of incubation, 20.0 µl of 5.0 mg/ml MTT reagent (Calbiochem, USA) was added into each well and mixed gently in shaker followed by 1 hr incubation in the dark, 37 °C until a purple formazan precipitate was clearly visible.

The spent media was then aspirated and 200.0 µl of DMSO was added to all the wells to dissolve the purple formazan precipitate and absorbance was measured at 570 nm

wavelength with a 650 nm reference wavelength using the Tecan Sunrise microtiter plate reader (Tecan, Switzerland) and quantification was carried out using the Magellan version 6.3 software (Tecan, Switzerland).

Assessment of the type of combination relationship was done using an isobologram analysis, while the type of interaction was determined based on combination index (CI) calculation using formula: CI = (D1c/D1) + (D2c/D2) + (D3c/D3) where D1, D2, and D3 are the doses for each drug/agent alone that inhibit 50 %, and D1c, D2c, and D3c are the doses for each drug/agent in a combination that inhibit the same 50 % (Koay *et al*., 2010). CI indicates additivity when CI=0.8–1.2; synergism when CI < 0.8; and antagonism when CI > 1.2.

3.11 *In vivo* animal model study

The 6-weeks old female BALB/c mice weighing 15-18 g were used in this tumour orthograft experiments and fed with sterilized food pellets and water. There were 7 experimental groups (placebo, single agents and combination groups), n=6. Induction of tumour was done by injecting suspensions of 100.0 µl of the 4T1 mouse mammary cells ($1x10^7$ cells/ml) in 1xPBS subcutaneously (s.c) at the mammary pad region using 25 gauge needles (Becton Dickenson and Co, USA). All drugs were prepared accordingly as shown in Table 3.4. Drugs were dissolved in 0.9 % (w/v) NaCl solution and administered via s.c. route locally at tumour induction sites once tumours reached 100.0 mm^3 in volume. Standalone and combination treatments were administered two times a week at 3 days intervals via *in situ* s.c injections and sterile PBS solutions were used as placebo controls. Tumour volumes were assessed by measuring (length x width x height) with a Traceable Digital Callipers (Thermo Scientific, USA) every 7 days post treatment and net body weight of tumours were measured. All animal studies were conducted in Animal

Ethics Unit, Faculty of Medicine, University of Malaya. Termination of tested mice was done using purified CO_2 gas according to the American Veterinary Medical Association (AVMA) guidelines on euthanasia. Approval from the Institutional Animal Care and Use Committee (IACUC) of Universiti Malaya (Reference number: 2015-181103/IBS/R/MS) was obtained prior to the commencement of the experiment. Post *in vivo* analysis was carried out by analysing samples from n=3 mice since mice from certain groups were terminated during the treatment course due to restriction in movement.

Table 3.4: Treatment groups and doses used for assessment of single, double and triple combinations of MIP, ACA and CDDP on *in vivo* BALB/c mice model

Treatment group	**Drug/Dose**			
	0.9 % NaCl (ml)	MIP (bacilli/mouse)	ACA (mg/kg)	CDDP (mg/kg)
Placebo	0.1	-	-	-
MIP	-	5×10^8	-	-
ACA	-	-	1.56	-
CDDP	-	-	-	10.0
MIP/ACA	-	5×10^7	0.78	-
MIP/CDDP	-	5×10^7	-	5.0
MIP/ACA/CDDP	-	5×10^7	0.78	5.0

3.11.1 Dehydration and paraffinization of tissue

Tumour biopsies were harvested, fixed in 10 % (v/v) neutral buffered formalin (NBF) (Merck, Germany) for 24 hr, then dehydrated by immersing in a graded alcohol series and followed by wax infiltration series. Once completely dehydrated, each sample were inserted into a small container containing molten paraffin wax and allowed to completely solidify. Formalin-fixed paraffin-embedded (FFPE) samples can be stored indefinitely.

3.11.2 Histopathological examination

Tumour, liver, spleen, kidney, lung, and heart were collected for histopathological examination to observe any systemic toxicity at the major organs. Organs and tumours were fixed in 10 % (v/v) neutral buffered formalin (NBF) (Merck, Germany) for 24 hr, followed with dehydration by immersing in a graded alcohol series and wax infiltration series. Then, routinely processed for paraffin embedding, sectioned at 5 μm thickness, stained with hematoxylin-eosin (H&E), and evaluated under a light microscope. Organ damage due to toxicity was assessed by pathologist at the University of Malaya's Department of Pathology. Histopathological evaluations were performed in accordance with the guidelines of the Society of Toxicologic Pathology. Images were taken using a Carl Zeiss Axio. All the microscopic images were captured using an AxioCam MRc5 CCD camera and all slides were reviewed and regraded for this study by the histo-pathologist, Dr. Mun Kein Seong.

3.12 Protein expression analysis

3.12.1 Immunohistochemistry

Immunohistochemistry procedure consisted of 5 parts: de-paraffinization, antigen unmasking, staining, dehydration and mounting. Firstly, de-paraffinization was carried out using 3 washes of xylene, 5 min each followed by 2 washes of 100 % and 95 % ethanol for 10 min each. Sections were washed with two times in distilled water for 5 min each before proceeding to antigen unmasking. Epitope retrieval was achieved by boiling the tissue sections in sodium citrate buffer (0.01 M, pH 6.0) for 10 min followed by cooling slides on bench top for 30 min. Endogenous peroxidase activity was blocked using 3 % (v/v) hydrogen peroxidase (Friedemann Schmidt, Francfort, Germany) and washed with distilled water. All sections were blocked with Tris Buffered Saline with Tween-20

(TBST) and 5 % (v/v) normal goat serum (Cell signaling, USA) for 1 hr at room temperature. Next, blocking solution was removed from sections, then incubated overnight with primary antibody diluted with TBST according to manufacturer's protocol (Table 3.5). Antibody solution was then removed and washed with TBST solution three times for 5 min each. Sections was covered with 1-3 drops of Signalstain® Boost Detection Reagent (HRP, Mouse/Rabbit) (Cell Signaling, USA) and kept in humidified chamber for 30 min at room temperature. The sections were washed and further developed with DAB solution (Sigma-Aldrich, USA). Counter staining was done using hematoxylin (Sigma-Aldrich, USA) and washed in distilled water. The sectioning were dehydrated by soaking in graded alcohol (90 % and 100 %) and cleared by soaking in xylene. Sectioning were mounted and cover-sliped using distyrene plasticizer and xylene (DPX) mounting medium (Thermo Scientific, USA). Images were captured using an inverted fluorescence microscope Nikon Eclipse TS 100 (Nikon Instruments, Japan) and quantified using the Nikon NIS-BR Element software (Nikon Instruments, Japan). Negative controls were also run in order to test the protocol and to support the validity of the staining.

Table 3.5: Summary of type, source and optimized dilution rate for antigen of primary antibodies used in IHC experiments

Antigen of Primary antibody	Source	Brand/Company	Dilution
NF-κB p65	Rabbit	Cell signaling	1:800
IκBα	Mouse	Santa Cruz	1:50
p-IκBα	Mouse	Santa Cruz	1:50
p300	Mouse	Santa Cruz	1:50
HDAC2	Mouse	Santa Cruz	1:100
Cleaved caspase 3	Rabbit	Cell signaling	1:2000
p21	Mouse	Santa Cruz	1:100
VEGF	Rabbit	Cell signaling	1:1600
COX-2	Rabbit	Cell signaling	1:600
CDK4	Mouse	Santa Cruz	1:50
MMP-9	Mouse	Santa Cruz	1:100
Cyclin D1	Rabbit	Cell signaling	1:50

3.13 Multiplex assay

Serum samples were prepared for analysis in a 96-well plate using Mouse Th17 Magnetic bead panel, MILLIPLEX® MAP kit to detect level of cytokines; IFN-ɣ, IL-6, IL-2, IL-10, IL-12p70 and TNF-α. In this way, multiple analytes within each test sample could be measured simultaneously. The concentrations of analytes were calculated by comparison to standard curves. The cytokines level was measured according to the manufacturer's instruction using a Luminex xMAP system (Luminex Corporation, 12212 Technology Blvd Austin, TX, USA). Standard calibration curves ranged from 7.8-8000 pg/ml for IFN-ɣ and IL-6; 6.9-6000 pg/ml for IL-2; 20-20000 pg/ml for IL-10 and IL-12p70; 3.4-3500 pg/ml for TNF-α.

Antibody-immobilized beads were prepared prior to adding serum sample. All six different antibody beads were vortexed for 1 min before 60 µl of each antibody beads were mixed in mixing bottle and brought to a final volume of 3.0 ml with Assay buffer. The mixed beads were vortexed. In a 96-well plate, 200 µl of Wash buffer were added into each well, then sealed with plate sealer prior to mixing on a plate shaker for 10 min at room temperature. Wash buffer were discarded and residual buffer on the plate were removed by inverting the plate and tapping it onto absorbent towels several time. A 25 µl of each standard and control added into appropriate well, followed by 25 µl of Assay buffer to the sample wells. Serum matrix solution (25 µl) was added to background, standard and control wells. Next, 25 µl of sample was added into the appropriate wells followed by 25 µl of mixed beads into each wells. Mixing bottle were vortexed intermittently to avoid settling of beads during this step. The plate was sealed with plate sealer and wrapped with aluminum foil and incubated with agitation on a plate shaker overnight at 4 °C. The following day, well contents were gently removed and plate was washed with 200 µl wash buffer. For the washing step, plate was rest on magnetic plate washer for 60 sec to allow complete settling of magnetic beads. The well contents then gently discarded by decanting the plate and gently tapping on absorbent pads to remove residual liquid. The plate was washed twice with 200 µl washing buffer by removing plate from magnet holder, adding wash buffer, shaking for 30 sec, reattaching to magnet, letting beads to settle for 60 sec and removing well contents. Upon washing, 25 µl of detection antibodies were added into each wells. The plate was sealed, covered and incubated with agitation on a plate shaker for 30 min at room temperature. Then, the well contents were gently removed and plate was washed twice as described previously. Sheath Fluid (150 µl) was added in each wells and the beads were re-suspended in a plate shaker for 5 min. Finally, the plates were read using Luminex 200^{TM} with xPONENT software.

3.14 Statistical analysis

All experiments were carried out in triplicates and presented as mean values ± standard deviation. Student's T-test was used to determine the statistical significance of results, where a *P* value of ≤ 0.05 was considered significant.

CHAPTER 4: RESULTS

4.1 MIP growth curve

Growth profile of *Mycobacterium indicus pranii*, MIP was plotted to identify the number of bacteria at specific OD. The growth profile was plotted for 20 days up to its stationary phase in Middlebrook 7H9-ADC medium (Figure 4.1). The resulting growth curve is sigmoidal in shape and has three different phases: lag (0-1 day), exponential or logarithmic (2-14 days) and stationary phase (14-20 days).

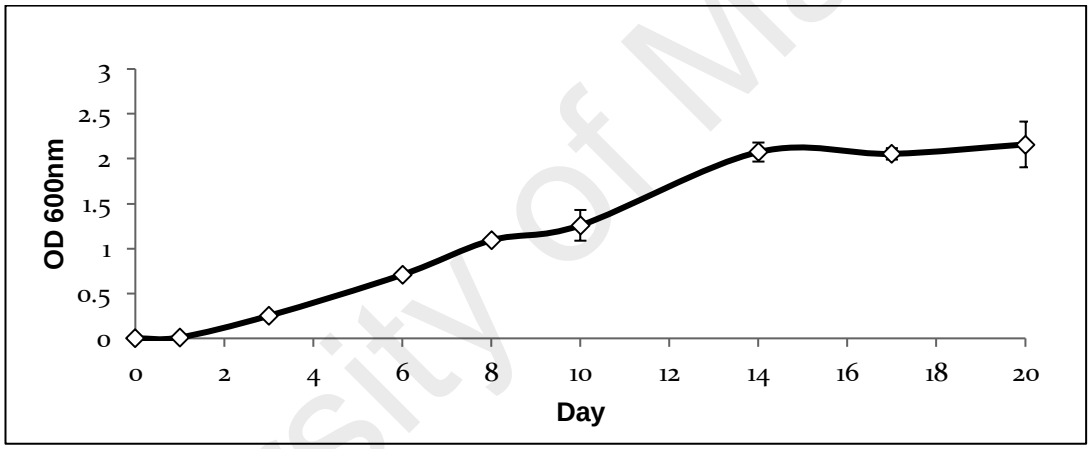


Figure 4.1: Growth rate analysis of MIP. MIP was cultured in Middlebrook 7H9-ADC medium at 37°C. The A_{600nm} of liquid culture of MIP was plotted against time to analyze the pattern of MIP growth. Growth was monitored by measuring the change in the value of A_{600nm} over time. Each experiment was performed with replicates and error bars for each time point are shown.

During the lag phase, cells adjust to their new environment, thus number of cells remains the same. The length of the lag phase varies due to temperature, inoculum size and medium (Montville & Matthews, 2001). As stated by a different study, lag phase also depends on the energy required by the cells to both adjust to a new environment and repair injury (Robinson *et al*., 1998). At exponential phase, cell doubling will occur where bacteria divides at a constant rate until the medium is eventually depleted of nutrients and

accumulated with wastes which inhibits bacteria growth and could even be toxic to bacteria. At stationary phase, the bacterial growth is equal to bacterial death rate, thus the curve is constant. Typically, the stationary phase is caused by high cell concentrations, low partial pressure of oxygen, and accumulation of toxic metabolic end products (Schlegel, 1992).

4.1.1 Bacteria CFU counting

Figure 4.1.1 shows standard curve comparing MIP cell number with OD 600nm to estimate number of bacterial cells present in the culture upon treatment. This is highly important to standardise number of bacteria when it comes to treating cancer or normal cells.

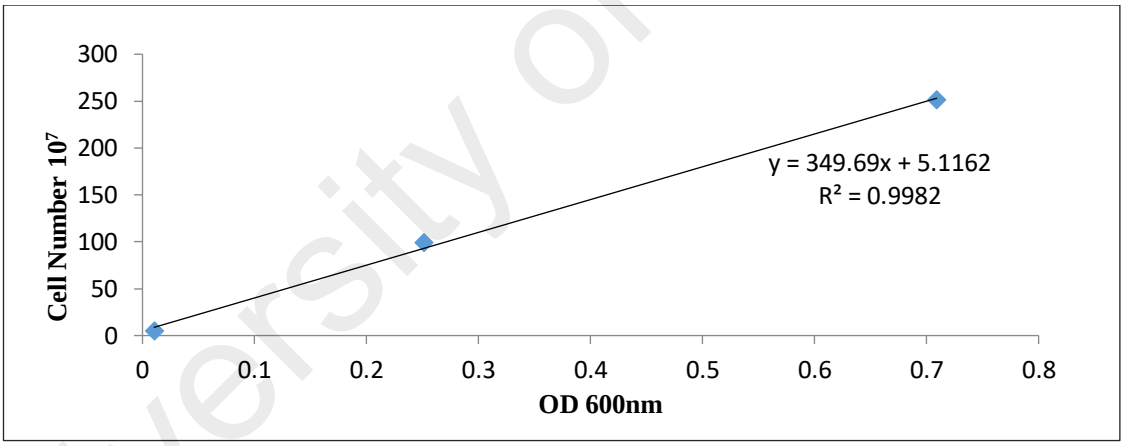


Figure 4.1.1: Standard curve comparing the OD 600nm of MIP broth with the number of viable cells/ml from standard plate count.

4.1.2 Identification of optimum temperature in preparation of heat killed bacteria

While past studies have cited autoclaving for 20 min at 15 lb/in^2 as the most common heat killing method (Purswani *et al*., 2011; Ahmad *et al*., 2011; Gupta *et al*., 2012), this method may denature important proteins, which led us to heat kill MIP using water bath to completely inactivate this bacterium. A preliminary test was carried out to identify optimum temperature to completely kill MIP using water bath. As shown in Figure 4.1.2,

temperature up to 50 °C was not sufficient in killing MIP as the colonies were observed after a week of incubation. While complete heat killing of MIP was observed at 60 °C and 70 °C as no growth was noticed on agar plate which confirms complete killing at these temperatures. This method is recommended as even though the bacteria were completely heat killed, the other intracellular and extracellular proteins/precursors potentially responsible for its cytotoxicity would remain intact. Therefore, heat killed MIP at 60 °C was carried out throughout this study.

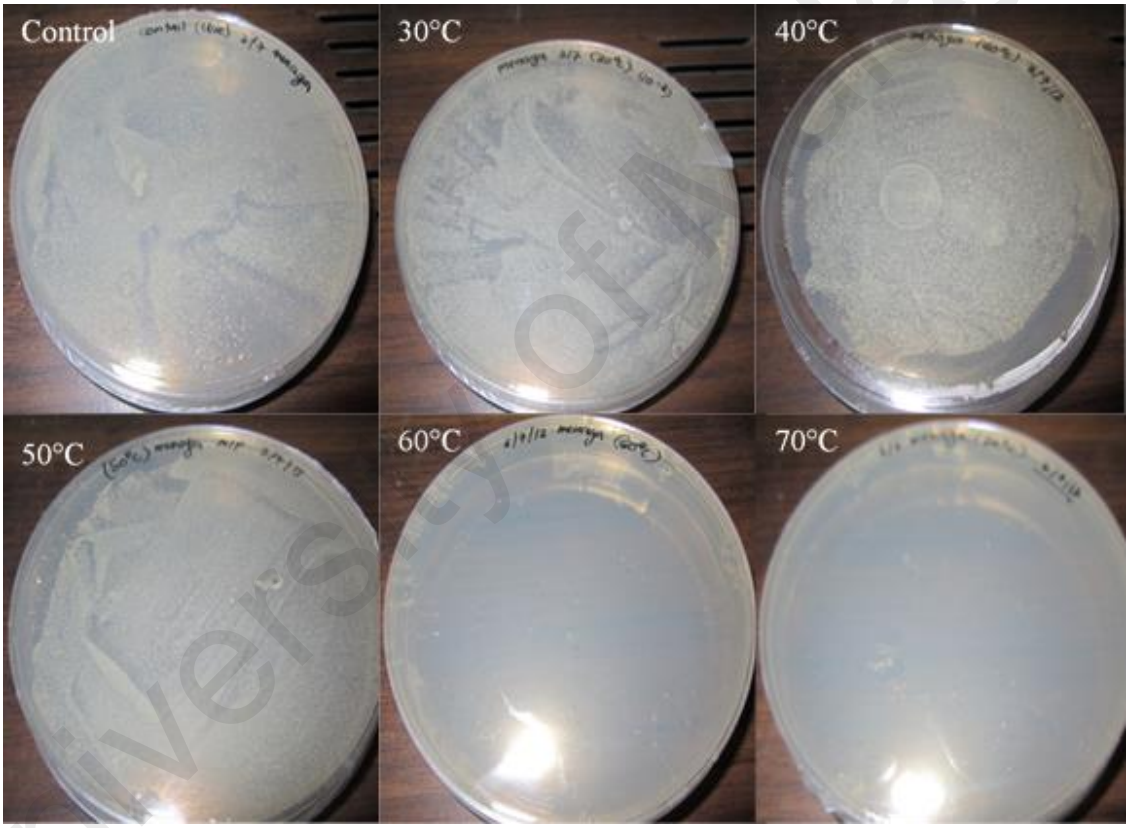


Figure 4.1.2: Growth of MIP upon heat killed at five different temperatures. Middlebrook 7H10 agar plate was supplemented with 10 % albumin-dextrose complex enrichment (ADC) and incubated at 37 °C for a week.

4.2 Agar diffusion assay

The agar diffusion method, also known as the Kirby Bauer Test, was developed in 1966 at the University of Washington and is still used in many clinical microbiology labs (Jorgensen & Ferraro, 2009). A known concentration of bacteria is plated onto agar plate before paper disc with antibiotic/drug was placed on the surface of the agar. The antibiotic/drug would passively diffused out into agar and inhibit the bacterial growth. Inhibition of bacterial growth can be evaluated qualitatively by observing the zone of inhibition. In this study, agar diffusion assay was carried out to verify safe usage of natural compound, ACA and commercial drug, CDDP together without inhibiting bacterial growth rate. As shown in Figure 4.2, CDDP and ACA treated plates did not produce zone of inhibition around the disc which indicated that CDDP and ACA at the tested concentrations are safe to be used together with MIP. Neomycin was used as a negative control, where it inhibits MIP growth and produces zone of inhibition from 3 to10 µg/ml in a dose dependent manner. These results shows MIP, ACA and CDDP can be used in combination without inhibiting bacterial growth.

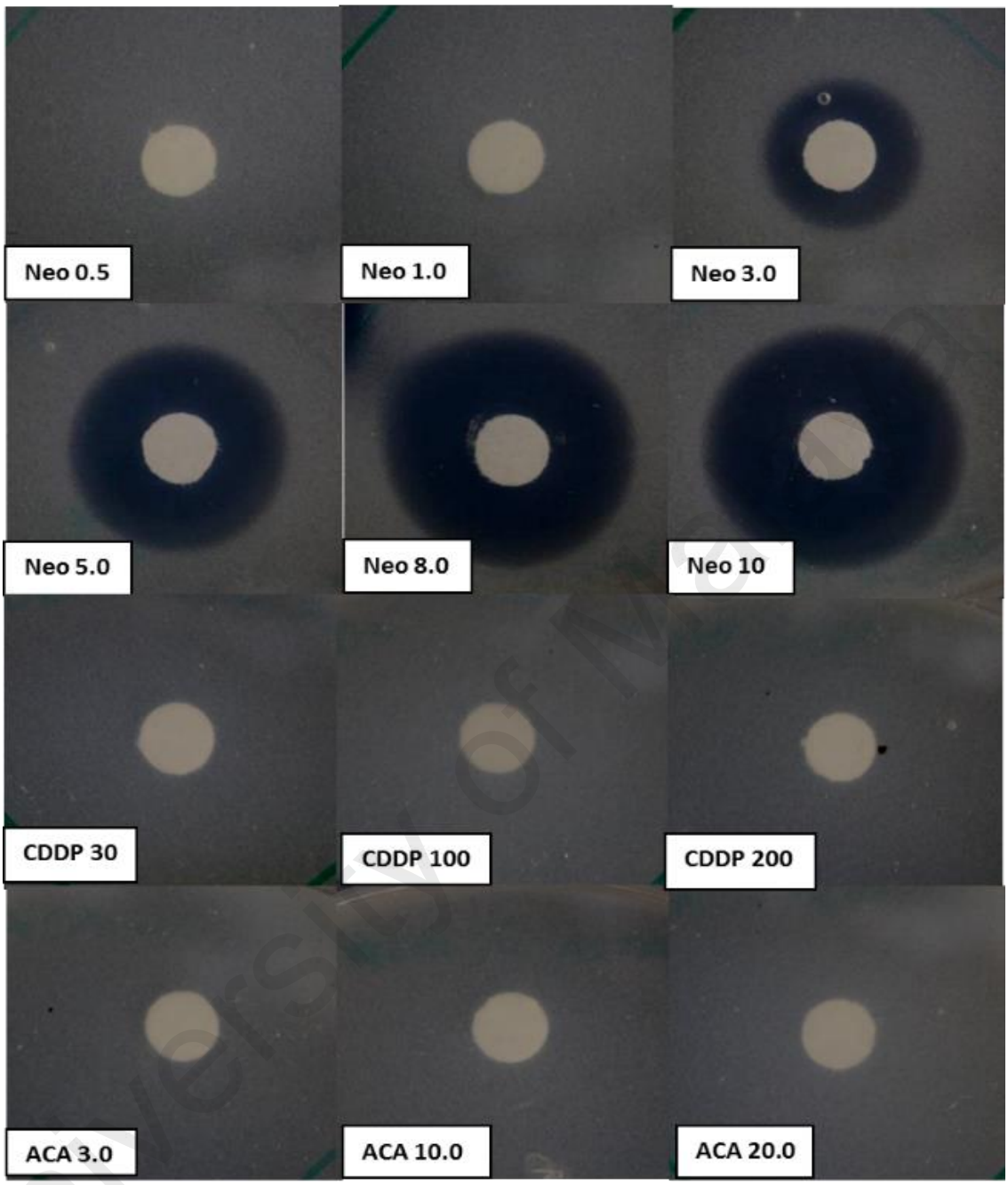


Figure 4.2: Disc-diffusion assay of MIP against ACA and CDDP with Neomycin as control. Middlebrook 7H10 agar plates of discs were impregnated with ACA (3.0, 10.0, and 20.0 mM), CDDP (30, 100 and 200 mM) and Neomycin (0.5, 1.0, 3.0, 5.0, 8.0 and 10.0 µg/ml).

4.3 MTT cytotoxicity assay

4.3.1 Cytotoxicity effects of MIP fractions on cancer cell lines

The MTT assay was used based on the mitochondrial activity in viable cells to test the cytotoxic and anti-proliferative effects of all four different MIP fractions randomly on the cellular viability of human lung, A549 and cervical cancer, CaSki cell lines. In addition, active MIP fraction was selected from this preliminary test based on its ability to inhibit cancer cells growth. As shown in Figure 4.3, among four MIP fractions, only heat killed bacteria (HKB) shows killing effect on A549 and CaSki in a dose dependent manner whereby heat killed supernatant (HKS), live bacteria (LB) and live supernatant (LS) does not affect cancer cells viability as it was maintained at 100 % even when the MIP dose was increased. Thus, throughout this study only HKB fraction was tested.

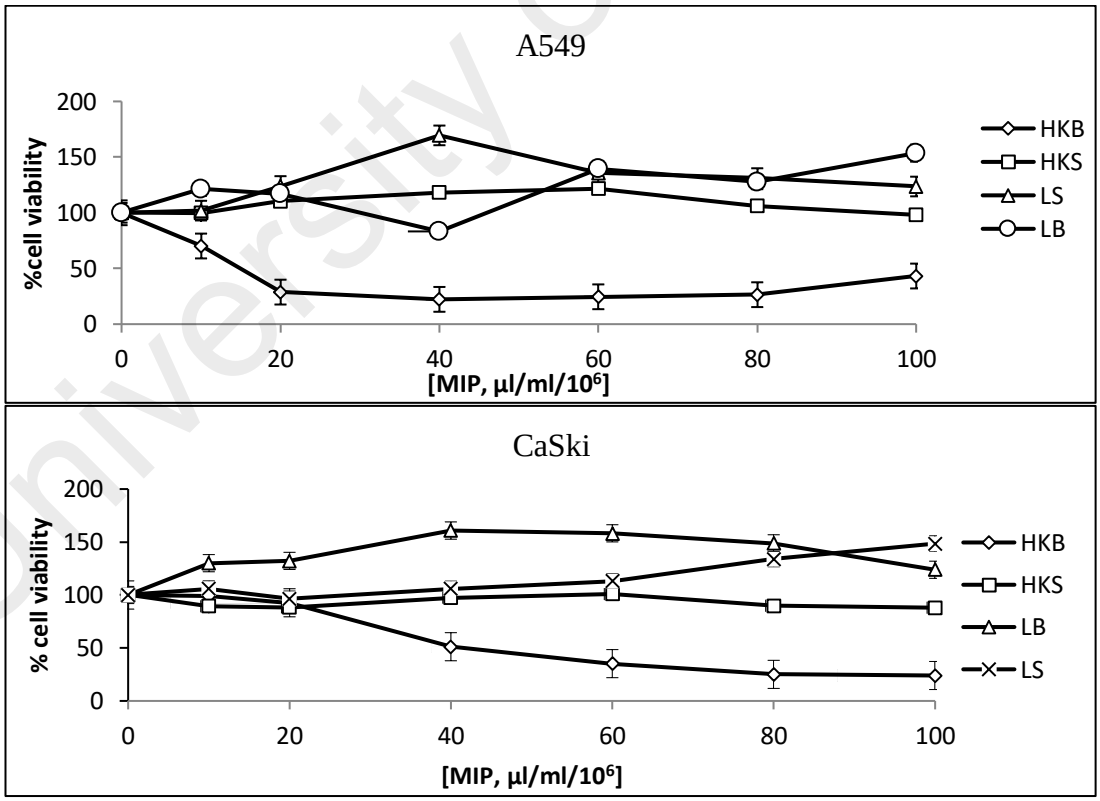


Figure 4.3: Cytotoxicity assay using MIP fractions at 24 hr in human cervical carcinoma cell line (CaSki) and human lung carcinoma cell line *(*A549). MIP fractions: live bacteria (LB), live supernatant (LS), heat killed bacteria (HKB) and heat killed supernatant (HKS). All MTT data were represented as mean ± SD of three independent experiments.

4.3.2 Cytotoxicity effects of heat killed bacteria (HKB) on cancer cell lines

Upon determining MIP active fraction through preliminary test, a complete MTT assay was carried out in seven different human cancer types: bladder (RT-112 and EJ-28); breast *(*MDA-MB-231 and MCF-7); liver (HepG2); prostate (PC-3 and DU-145); cervical (CaSki, and HeLa S3); lung *(*A549 and SK-LU-1) and oral (ORL-48, ORL-115 and ORL-136) at 24 hr as shown in Figure 4.4, MTT data obtained was also used to determine specific IC_{50} values. IC_{50} value is the concentration of drug required to kill 50 % of the cell population and was summarized in Table 4.1. As shown in Figure 4.4, cytotoxicity of MIP HKB is dose dependent and reached almost less than 40 % cell viability at 100 µl/(1.0×10^6 MIP cells/ml). Baseline killing of cancer cells was also achieved from 20.0 µl/(1.0×10^6 MIP cells/ml). Highest cytotoxicity was observed in liver cancer cell, HepG2 with an IC_{50} of 5.6 µl/(1.0×10^6 MIP cells/ml) at 24 hr. Oral cancer cells showed the second highest cytotoxicity (ORL-48, ORL-115 and ORL-136), with IC_{50} values of 13.6 µl/(1.0×10^6 MIP cells/ml), 7.8 µl/(1.0×10^6 MIP cells/ml) and 5.9 µl/ (1.0×10^6 MIP cells/ml), respectively followed by lung and breast cancers. HaCaT, MCF-10A and NP-69 with IC_{50} values of 23.5 µl/(1.0×10^6 MIP cells/ml), 25.7 µl/(1.0×10^6 MIP cells/ml) and 32.9 µl/(1.0×10^6 MIP cells/ml) respectively, implies that concentrations higher than these values are toxic to non-cancerous cells. Also IC_{50} values higher than 23 µl/(1.0×10^6 MIP cells/ml) in several cancer cells (PC-3, 34.5 µl/(1.0×10^6 MIP cells/ml); EJ-28, 51.9 µl/(1.0×10^6 MIP cells/ml); RT-112, 35.5 µl/(1.0×10^6 MIP cells/ml) indicates heat killed MIP treatment was less effective in these cancer cells.

4.3.3 Cytotoxicity effects of cisplatin (CDDP) on cancer cell lines

Cytotoxicity effect of CDDP was tested on seven different human cancer types as shown in Figure 4.5. Cytotoxicity was dose dependent and reached almost less than 50 % cell viability at 150 µM in all cancer type except in lung cancer cell line where cell viability was maintained above 50 % after 24 hr treatment in SK-LU-1 while in A549 cells highest IC_{50} reached about 92 µM. CDDP was sensitive in bladder cancer with 14.8 µM and 11.02 µM in EJ-28 and RT-112 cell lines, respectively. In both breast and cervical cancer types, moderate cytotoxicity was induced upon CDDP treatment with IC_{50} ranging from 34 µM to 66 µM.

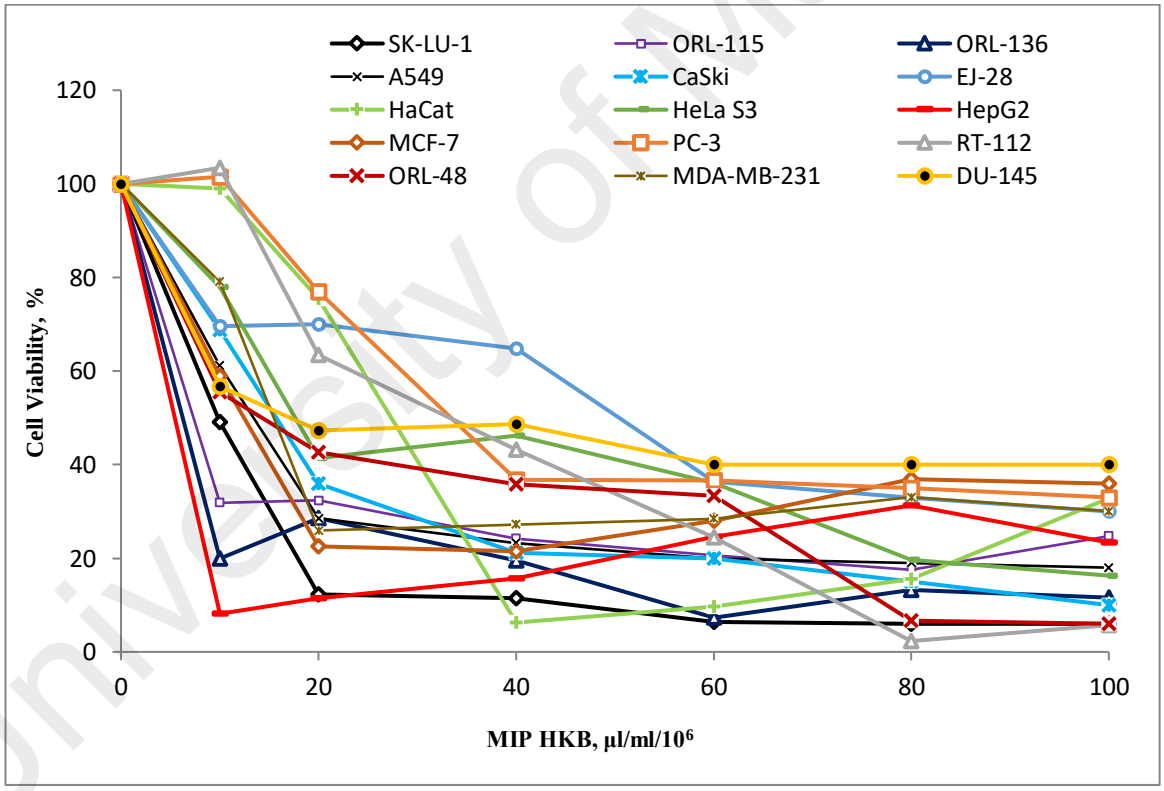


Figure 4.4: Cytotoxicity of MIP heat killed bacteria at 24 hr in various human cancer cell lines by MTT assay. Human bladder cancer cell lines (RT-112 and EJ-28); Human breast cancer cell lines (MDA-MB-231 and MCF-7); Human liver carcinoma cell line (HepG2); Human prostate cancer cell lines (PC-3 and DU-145); Human cervical carcinoma cell lines (CaSki, and HeLa S3); Human lung carcinoma cell lines (A549 and SK-LU-1); Human oral cancer cell lines (ORL-48, ORL-115 and ORL-136); Immortalized human keratinocyte cell line (HaCaT). Data is shown as mean ± S.D. of three independent replicates.

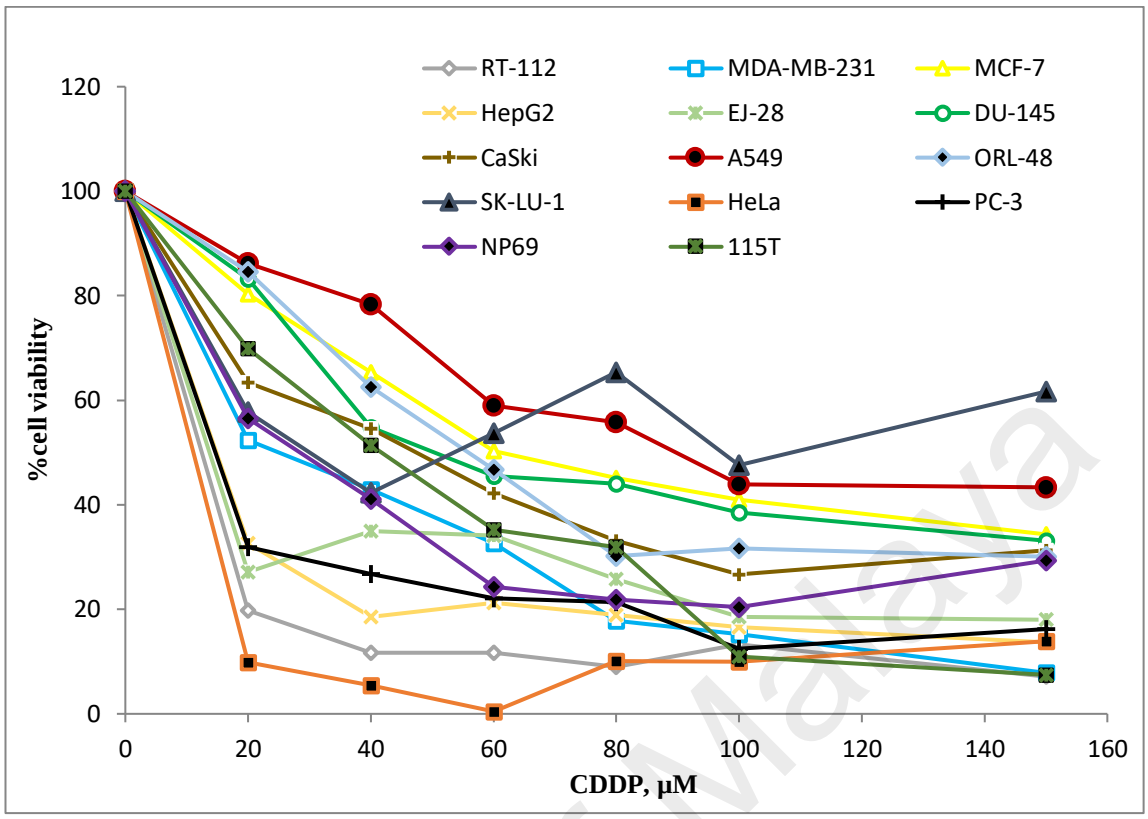


Figure 4.5: Cytotoxicity of CDDP at 24 hr in various human cancer cell lines by MTT assay. Human bladder cancer cell lines (RT-112 and EJ-28); Human breast cancer cell lines *(*MDA-MB-231 and MCF-7); Human liver carcinoma cell line (HepG2); Human prostate cancer cell lines (PC-3 and DU-145); Human cervical carcinoma cell lines (CaSki, and HeLa S3); Human lung carcinoma cell lines (A549 and SK-LU-1)*;* Human oral cancer cell lines (ORL-48, ORL-115 and ORL-136); Immortalized human keratinocyte cell line (HaCaT). Data is shown as mean ± S.D. of three independent replicates.

4.3.4 Cytotoxicity effects of ACA on cancer cell lines

The study on cytotoxicity effects of ACA on cancer cell lines was carried out by a previous student, thus, the data is presented in Table 4.1 as a comparison (Awang *et al.*, 2010). Based on IC_{50} values, the reduction on cellular viability for ACA was found to be greatest in bladder, liver and cervical cancer cell lines as IC_{50} values are lesser than 20 µM.

Evaluation of ACA effects on all cancer cells tested in this study also demonstrated a dose dependent cytotoxicity pattern similar to MIP HKB and CDDP. Dose-dependent cytotoxicity is an important characteristic of an anti-cancer drug. This means that the

administration of drugs could be easily and non-stringently manipulated since comparable levels of cytotoxicity can be achieved by switching IC values. Furthermore, systemic or physiological side effects due to high dose or prolonged treatment regime can be reduced.

Table 4.1: IC_{50} values of MIP, ACA, and CDDP standalone cytotoxicity effect on various human cancer cell lines

Cancer type	Cell Lines	CDDP (µM)	MIP HKB (MIP cells/ml)	ACA (µM)
Breast	MDA-MB-231	41.6±23.4	15.4±0.1	4.8±0.4
	MCF-7	63±2.3	12±0.7	30±0.3
Cervical	Caski	51.9±6.4	15.9±1.8	13±0.7
	SiHa	66±1.3	47.3±7.6	4.5±0.3
	HeLa S3	34.3±3.5	21.1±2.2	12±0.6
Lung	A549	92.2±1.5	14.3±1.3	26.5± 6.2
	SK-LU-1	n/a	7.8±2.8	26.7±0.7
Prostate	PC-3	10.89±0.8	34.50±1.6	26.7±2.3
	DU-145	44.1±8	18.4±1.7	19.5±2.9
Liver	HepG2	13±0.5	5.6±0.2	18±0.8
Bladder	EJ-28	14.8±.4	51.9±2	8.2±0.9
	RT-112	11.02±0.1	35.53±3.2	14.1±3.8
Oral	ORL-48	54.8±1.5	13.6±1	25.2±1.1
	ORL-115	39.8±2.7	7.8±1	7.3±1.5
Normal cells	HaCat	80.2 ± 4.6	23.5±5.4	n/a
	NP-69	n/a	32.9±1.0	n/a
	MCF-10A	n/a	25.7±0.6	n/a

n/a - not applicable as cell viability was maintained above 50 % after 24 hr treatment.

4.3.5 Cytotoxicity effects of double combination on various cancer cell lines

4.3.5.1 MIP/ACA double combination

Since MIP and ACA were found to induce cytotoxicity in a dose dependent manner in all the tested cell lines, we next sought to determine whether both these compounds could work in combination to enhance cytotoxicity and reduced drug dosage. MTT assay was used again to assess combinatory effect against various cancer types. Various concentrations of these compounds were tested to obtain optimum concentration ratio for the synergistic killing of cancer cells.

The effects of various cytotoxicity levels induced by ACA, in combination with MIP in seven cancer types at 24 hr are summarized in Table 4.2. It was observed that combining the effects of both MIP and ACA was successful in increasing the overall cytotoxicity level in all the cancer cells with reduction in IC_{50} values compared to standalone treatment of MIP and ACA. IC_{50} of MIP in breast cancer cell line, MCF-7, decreased from 15.4 µl/(1.0×10^6 MIP cells/ml) to 0.76 µl/(1.0×10^6 MIP cells/ml) when ACA was held constant at its IC_{25}. Similar patterns were observed in all cells tested where this reduction in IC_{50} values indicated the potentiating ability of MIP in combination with ACA. However, in oral cancer cell line ORL-115, this synergy pattern was not achieved as the combination of MIP and ACA did not exert 50 % killing of cell population due to the moderate drug dosage used. This concentration need to be increased (such as using IC_{50} of ACA or increasing MIP concentration) to obtain 50 % cell killing of ORL-115. Overall, MIP/ACA combination showed increased cytotoxicity with reduction in each drug concentrations.

4.3.5.2 MIP/CDDP double combination

As a follow-up to MIP/ACA double combination, the cytotoxicity effect of MIP/CDDP with a similar reduced drug dosage was tested in all the cancer cell lines. MTT assay was used to investigate combinatory effect against various cancer types. Various concentrations of these compounds were tested to obtain optimum concentration ratio for the synergistic killing of cancer cells.

The effects of various cytotoxicity levels induced by CDDP, which is a common synthetic platinum-based pyrimidine analog capable of interfering with DNA replication and frequently employed as an anticancer drug against cervical cancer were also evaluated for its combinatorial effects with MIP. The combination was tested in seven cancer types at 24 hr and summarized in Table 4.2. It was observed that combining the effects of both MIP and CDDP was successful in increasing the overall cytotoxicity level in all the cancer cells with reduction in IC_{50} values compared to standalone treatment of MIP and CDDP. IC_{50} of MIP was decreased in combination when CDDP was held constant at its IC_{25} in all cancer cells tested where this significant reduction in IC_{50} suggested the potentiating ability of MIP in combination with CDDP. However, in ORL-115, DU-145, SK-LU-1 and CaSki this reduction was not seen since combination of MIP and CDDP does not exert 50 % killing of cell population due to moderate drug dosage. This concentration need to be increased (such as using IC_{50} of CDDP or increasing MIP concentration) to obtain 50 % cell killing. Generally, MIP/CDDP combination in most of the cancer cell lines tested showed increased cytotoxicity with reduction in each drug concentration.

Table 4.2: MIP/ACA and MIP/CDDP double combination treatment at 1:1 ratio on various human cancer cell lines

Cancer type	Cell line	MIP/ACA			MIP/CDDP		
		IC_{50}	CI	Relation	IC_{50}	CI	Relation
Breast	MCF-7	0.76±0.2	0.58	S	0.72±0.1	0.4	S
	MDA-MB-231	1.8±0.1	0.7	S	4.4±0.7	0.4	S
Cervical	CaSki	1.4±0.2	0.9	AD	n/a	n/a	n/a
	SiHa	4.1±0.2	0.3	S	2.9±0.3	0.2	S
	HeLaS3	2.7±0.7	0.2	S	1.3±0.1	0.2	S
Lung	A549	1.2±0.2	0.7	S	2.7±1.0	0.9	AD
	SK-LU-1	1.6±0.1	1.1	AD	n/a	n/a	n/a
Prostate	PC-3	3.3±0.1	1.1	AD	3.3±0.2	0.6	S
	DU-145	1.4±0.2	0.7	S	n/a	n/a	n/a
Liver	HepG2	0.3±0.2	0.7	S	0.32±0.1	0.6	S
Bladder	EJ-28	4.4±0.2	0.5	S	10.7±2.7	0.71	S
	RT-112	2.4±0.1	0.8	AD	2.6±0.4	0.57	S
Oral	ORL-48	1.7±0.3	0.3	S	2.3±0.5	0.4	S
	ORL-115	n/a	n/a	n/a	n/a	n/a	n/a

n/a - not applicable; CI-combination index; S-synergistic; AT-antagonistic; AD-additivity
* $IC_{50,}$ (µl/(1.0 x 10^6 MIP cells/ml) shows amount of MIP required to achieve 50 % cell killing at constant ACA or CDDP

4.3.5.3 MIP/ACA/CDDP triple combination

Triple combination of MIP, ACA and CDDP was tested to investigate its cytotoxicity effects against various cancer types at 24 hr and summarized in Table 4.3. Triple combination was carried out with ACA and CDDP kept at constant IC_{25} while MIP was tested in increasing dosage up to IC_{25} which gives ratio of 1:1:1 (IC_{25}:IC_{25}:IC_{25}). As expected, 50 % cell killing was obtained in all cancer cells with reduced IC_{50} compared to standalone. However, the IC_{50} values were not reduced in all the tested cell lines compared to double combinations. The IC_{50} was not decreased in oral cancer cell lines, ORL-115 and ORL-48 and remained the same in HepG2 liver cancer cell line.

Triple combination was also repeated in lower doses using IC_{10} in a ratio of 1:1:1 for all three agents. Interestingly, 50 % cell killing was obtained at this lower dose in breast, cervical, prostate, liver, bladder and oral cancer cell lines. Moreover, the 50 % killing using the IC_{10} combination was reduced significantly compared to the IC_{25} combination, which indicated that reduced dosages of the triple combination was able to give the same cytotoxicity as higher dosages. However at these reduced dosages, cytotoxicity of 50 % killing was not achieved in some cancer cell lines, namely, A549, SK-LU-1, EJ-28 and ORL-115.

Table 4.3: MIP/ACA/CDDP triple combination treatment on various human cancer cell lines

Cancer type	Cell line	MIP/ACA/CDDP (25:25:25)			MIP/ACA/CDDP (10:10:10)		
		IC_{50}	CI	Relation	IC_{50}	CI	Relation
Breast	MCF-7	0.6±0.4	0.92	AD	0.3±0.3	0.41	S
	MDA-MB-231	1.8±0.1	1.0	AD	1.3±0.2	0.44	S
Cervical	CaSki	1.2±0.1	1.1	AD	2.5±0.5	0.82	AD
	SiHa	2.0±0.7	1.6	AT	3.2±0.2	0.4	S
	HeLa S3	1.5±0.1	1.3	AT	0.5±0.3	0.7	S
Lung	A549	1.4±0.1	1.7	AT	n/a	n/a	n/a
	SK-LU-1	1.4±0.1	1.9	AT	n/a	n/a	n/a
Prostate	PC-3	4±0.13	1.7	AT	2.9±0.1	0.48	S
	DU-145	1.4±0.1	1.5	AT	12±1.6	1.1	AD
Liver	HepG2	0.3±0.3	1.2	AD	0.2±0.1	0.6	S
Bladder	EJ-28	3.5±0.4	1.0	AD	n/a	n/a	n/a
	RT-112	2.0±0.2	1.3	AT	3.5±0.6	0.6	S
Oral	ORL-48	4.4 ±0.1	1.1	AD	0.5±1	0.4	S
	ORL-115	3.0±0.1	1.4	AT	n/a	n/a	n/a

n/a - not applicable; CI - combination index; S - synergistic; AT - antagonistic; AD -additive
* $IC_{50,}$ (µl/(1.0 x 10^6 MIP cells/ml) shows amount of MIP required to achieve 50% cell killing at constant ACA or CDDP

4.4 Combination index analysis

Despite current observations indicating that combinations of ACA, MIP and CDDP in double and triple combinations with reduced IC_{50} levels, it was necessary to identify the type and extent of combinatory interactions involved. It has been shown that two/ three drugs that produce clearly similar effects will sometimes produce exaggerated or diminished effects when used concurrently. Therefore, a quantitative assessment was necessary to distinguish these cases from a simply additive action. We evaluated the *in vitro* cytotoxicity of anticancer drug combinations using the median-effect analysis method of Chou & Talalay. (1984), where the measure of synergy is defined by the Combination index (CI) value. CI analysis is a popular method for evaluating drug interactions in combination cancer chemotherapy. CI formula was used to calculated CI value, where CI indicates additivity when CI=0.8–1.2; synergism when CI < 0.8; and antagonism when CI > 1.2.

In breast cancer cell lines MCF-7 and MDA-MB-231, synergistic effect was observed in MIP/ACA and MIP/CDDP combinations when ACA or CDDP was held constant. CI reached 0.4 in both breast cancer cell lines in MIP/CDDP combination. While in triple combination, additive relationship was seen in both cells at IC_{25} combination which showed similar effect of drug obtained as in standalone treatment. However, synergism was achieved at lower dose of IC_{10} with CI 0.4 in both breast cancer cell lines.

In cervical cancer, both MIP/ACA and MIP/CDDP double combinations gave synergistic effects in SiHa and HeLa S3, while additivity was observed in CaSki when treated with MIP/ACA. However 50 % cytotoxicity was not achieved when treated with MIP/CDDP combination. In IC_{25} triple combination, additive effect was only observed in CaSki and it remained at lower dose of IC_{10}. This showed MIP does not potentiate cytotoxicity effect

of ACA and CDDP in combination against CaSki cells. While in SiHa and HeLa S3 cervical cancer cell lines, lower doses showed a shift from antagonism to synergism, where all the drugs work together to give increased effect in comparison to standalones.

In A549 lung cancer cell line, synergistic interaction was not observed in MIP/ACA combination while additive effects were seen in MIP/CDDP double combination. This showed MIP/CDDP combination gave similar effect of cytotoxicity as in standalone treatment of MIP and CDDP. Triple combination of ACA and CDDP did not potentiate MIP to induce synergistic effects in IC_{25} combination and reduced dose at IC_{10} did not exert 50 % of cell killing. In SK-LU-1, MIP/ACA gave additive effect and MIP/CDDP did not achieved 50 % cell killing. In triple combination, antagonistic effect exerted at IC_{25} combination and 50 % cell death was not reached at IC_{10} combination. Overall, double and triple combinations of the three agents showed poor effects or interaction against lung cancer cells.

Similar to lung cancer, MIP/ACA/CDDP triple combination showed poor interaction in prostate cancer cell lines, PC-3 and DU-145, where synergistic effects was only observed in IC_{10} combination against PC-3 cells. In double combination, MIP/ACA gave synergistic interaction in DU-145 with CI value of 0.7 while MIP/CDDP exerted synergistic effects with CI 0.6 against PC-3 cells. Next, in HepG2 liver cancer cell lines, MIP, ACA and CDDP combinations showed promising interaction where synergism was achieved in both double and triple combinations at lower dose of IC_{10}. Double combination in bladder cancer showed promising effect where synergistic effects exerted in EJ-28 for MIP/ACA and MIP/CDDP combinations while in RT-112 synergistic effect observed during MIP/CDDP treatment. However, in triple combination MIP does not

potentiate synergistic effect at IC_{25} combination in both cells while at IC_{10} synergism was only observed in RT-112.

A poor interaction was also obtained in oral cancer cells. In ORL-115, double combination of MIP/ACA and MIP/CDDP failed to achieve 50 % cell death, thus it was unable to obtain IC_{50} value. In triple combination, antagonistic effect was observed in MIP/ACA combination while reduced dose did not achieved 50 % cell killing. However, in ORL-48, synergism was achieved in MIP/ACA and MIP/CDDP combinations while in triple combination interaction is shifted from additive to synergism when dosage was changed from IC_{25} to IC_{10}.

Overall, MCF-7 breast cancer cell line was selected for the following studies since it showed both synergistic interactions in double and triple combinations compared to the other cancer types.

4.5 Mode of action of heat killed bacteria

Prior to investigating the mode of cell death upon combination treatment, mode of action of heat killed bacteria was examined. It is important to identify cell death caused by HKB fraction since we have used a novel method to prepare MIP HKB in this study whereby it is inactivated by heat at 60 °C. Breast cancer cell line, MCF-7 and oral cancer cell line, ORL-115 were randomly selected as model cell lines owing to possessing IC_{50} values lower than the HaCat cell line threshold.

The morphological changes in both cells showed MIP induced apoptotic cell death at 6 and 12 hr compared to 0 hr. The morphological changes included membrane blebbing,

cell shrinkage, nucleus fragmentation, chromatin condensation and DNA degradation. Occurrence of apoptotic cell death was confirmed by PARP and DNA fragmentation assay.

The PARP cleavage assay which measures the enzymatic cleavage of PARP following caspase activation was carried out to validate the apoptosis mediated cell death in both cell lines. PARP is a 116 kDa nuclear poly (ADP-ribose) polymerase, appears to be involved in DNA repair in response to environmental stress (Satoh & Lindahl, 1992). Caspases plays an important role in PARP cleavage both in *in vitro* and *in vivo* (Lazebnik *et al*., 1994; Cohen, 1997). Cleavage of PARP protein occurs between Asp 214 and Gly 215, which separates the PARP amino-terminal DNA binding domain (24 kDa) from the carboxy-terminal catalytic domain (89 kDa). Evaluation of PARP cleavage levels represents cellular disassembly and generally serves as a marker of cancer cells undergoing apoptosis (Oliver *et al*., 1998).

Cells were treated with PBS and MIP HKB, 12 µl/(1.0×10^6 MIP cells/ml) for MCF-7 while 7.8 µl/(1.0×10^6 MIP cells/ml) for ORL-115 in a time dependent manner at 6 and 12 hr to observe the initiation and progression of apoptosis. Figure 4.6 showed the cleavage of the inhibitory fragment from 116 kDa full length PARP into an 89 kDa fragment at 12 hr post treatment only in MIP treated cells. The housekeeping gene, GAPDH was used as a protein normalization and loading control. These western blotting results confirmed the occurrence of apoptosis mediated cell death induced by HKB on human breast and oral cancers *in vitro.*

Next, DNA fragmentation assay was carried out to confirm and observe the occurrence of late apoptosis in MCF-7 and ORL-115 cells in a time dependent manner. One of the

major hallmarks of apoptosis-mediated cell death is the occurrence of chromatin condensation and laddering. These characteristic occurs following the activation of endonucleases, which will then mediate nucleosome excision to smaller fragments of DNA of about 180-200 bp in length. A 150 bp to 200 bp laddering of DNA at 12 hr upon MIP HKB exposure in MCF-7 indicated a strong hallmark of late apoptotic events (Fig. 4.7). Ladder formation was absent in both untreated and PBS treated cells, which showed that the appearance of apoptotic DNA fragments were due to the cytotoxic effect of MIP HKB treatment. A positive control was also indicated in Figure 4.7, consisting of MCF-7 undergoing apoptosis upon treatment with 1'S-1'-acetoxychavicol acetate, ACA (Awang *et al*., 2010).

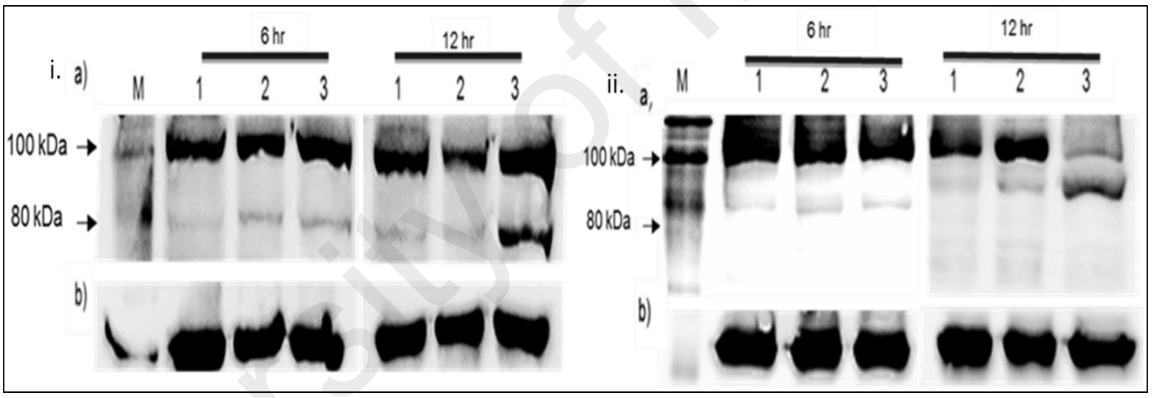


Figure 4.6: Effects of MIP HKB on PARP cleavage at 6 and 12 hr. i. MCF-7 cell line; ii. ORL-115 cell line. (a) Cells were treated with MIP HKB at 6 hr and 12 hr and PARP was measured by the western blot analysis. (b) GAPDH was used as a loading control. Lane M: biotinylated protein ladder; Lane 1: untreated cells; Lane 2: PBS treated cells; Lane 3: MIP HKB treated cells.

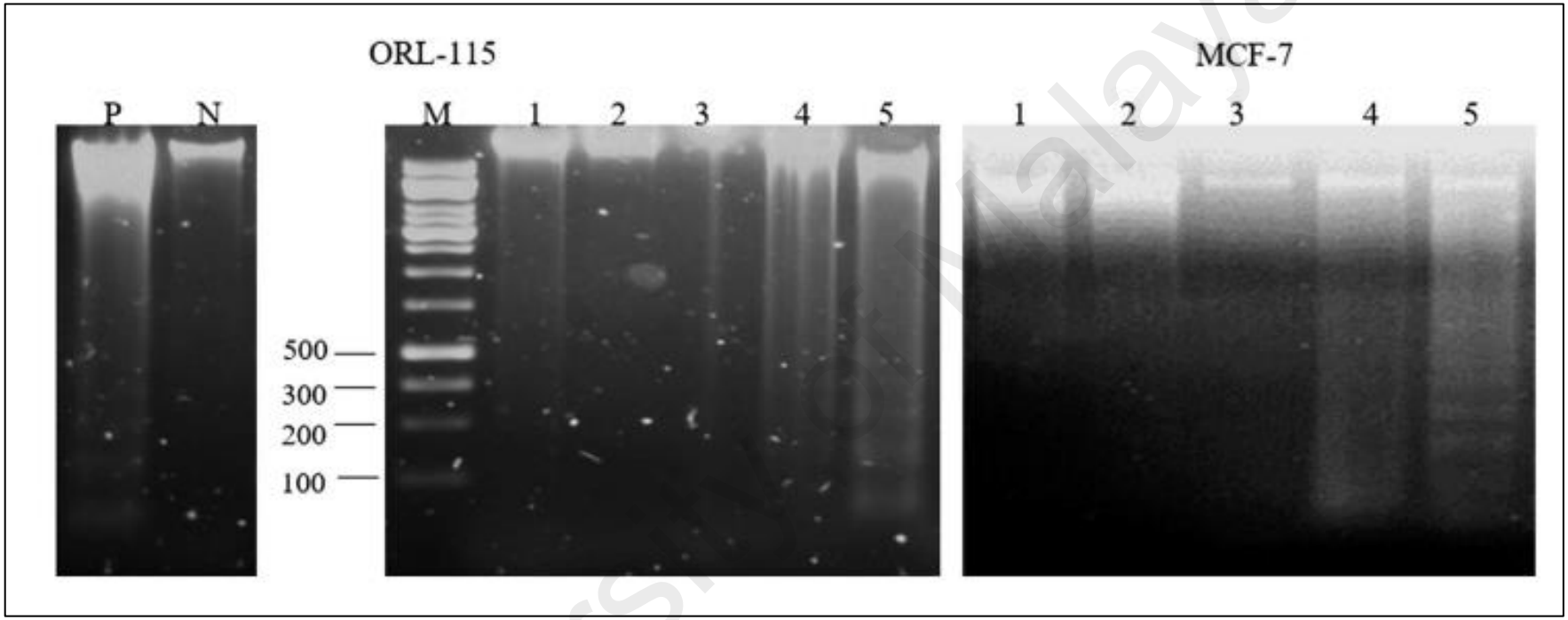


Figure 4.7: DNA gel electrophoresis of inter-nucleosome DNA fragmentation in 1.5 % agarose gel at 6, 12 and 24 hr treatment in MCF-7 and ORL-115 cell lines. Lane P: positive control; Lane N: negative control; Lane M: DNA molecular weight marker; Lane 1: untreated cells; Lane 2: PBS treated cells; Lane 3: HKB treated at 6 hr; Lane 4: HKB treated at 12 hr; Lane 5: HKB treated at 24 hr. DNA laddering was demonstrated in cells treated with MIP HKB in Lane 5.

4.6 Western blotting analysis on drug combination

4.6.1 The combination of MIP, ACA and CDDP activates intrinsic apoptosis

Apoptosis can be executed through two basic signalling pathways: the extrinsic and the mitochondrial intrinsic pathway. Caspase 3 is the most important of the executioner caspases, and is activated by any of the initiator caspases 8, 9 and 10 in both extrinsic and intrinsic pathways. Previous study has demonstrated that MIP could induce mitochondria-mediated apoptosis in mouse peritoneal macrophages *in vitro* (Pandey *et al*., 2011). The intrinsic pathway is activated by various stimuli like viral infection, DNA damage and absence of certain growth factors, hormones and cytokines. These stimuli lead to permeabilization of mitochondrial membrane, formation of pores, and release of cytochrome-c and other pro-apoptotic proteins into the cytosol. In the cytosol, cytochrome-c binds to apoptotic protease-activating factor-1 (Apaf-1), which in turn binds to pro-caspase 9 to form a complex known as the apoptosome. Binding to Apaf-1 induces conformational change and activation of caspase 9, which proteolytically activates executioner caspase 3 (Schmitz *et al*., 2000; Sharma *et al*., 2000). Thus, in this study intrinsic apoptotic protein expression in MIP double and triple combinations were tested using Apaf-1 and caspase-9 to validate the occurrence of intrinsic apoptosis mediated cell death upon combination with ACA or/and CDDP.

We observed that application of MIP, ACA and CDDP in double and triple combinations induced the release of Apaf-1 from 0 to 6 hr demonstrating that MIP/ACA, MIP/CDDP and MIP/ACA/CDDP activated the mitochondrial pathway (Figure 4.8). The level of Apaf-1 expression is maintained in MIP/ACA treated cell from 0 to 6 hr. Significant Apaf-1 increment was observed in MIP/CDDP treated cells at 3 hr while a significant decrease was seen in triple combination at 3 hr post treatment.

Intrinsic apoptosis occurrence also validated as shown in Figure 4.8 where caspase-9 cleavage products were observed in increasing concentrations, while procaspase 9 was concomitantly decreased upon treatment with all drugs/agent combinations at 6 hr in MCF-7. The housekeeping gene, GAPDH was used as a protein normalization and loading control.

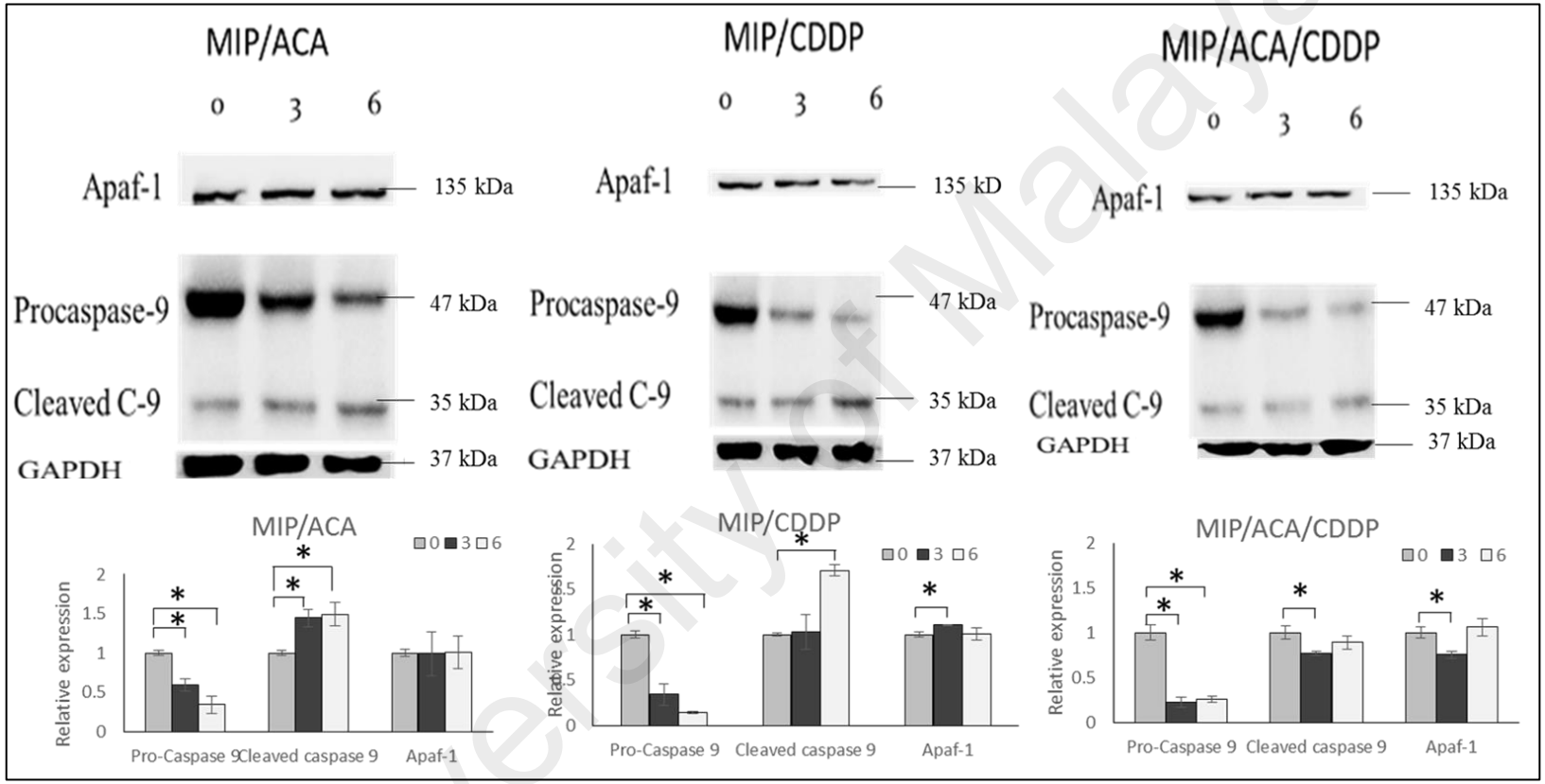


Figure 4.8: MIP, ACA and CDDP combination stimulated intrinsic apoptosis. MCF-7 cells were treated with MIP/ACA, MIP/CDDP and MIP/ACA/CDDP at 3 and 6 hr followed by Western blot analysis. Normalized quantification of proteins was performed on double and triple treatments. All band intensities were quantified and normalized against GAPDH using the ImageJ v1.43u software, and presented as mean ± SEM of three replicates.

4.6.2 The combination of MIP, ACA and CDDP inactivated NF-κB protein expression

Several studies have shown that, chemo-resistance is often contributed by the activation of nuclear factor kappa-B (NF-κB) by chemotherapeutic agents (Nakanishi & Toi, 2005). Thus a strategic approach to tackle cancer development is to formulate anticancer drug which targets NF-κB suppression. Previous microarray global expression study on ACA has shown that, a large portion of genes affected in oral squamous cell carcinoma were found to be either directly or indirectly related to the NF-κB pathway, corresponding to 88 % of the top 50 genes by fold change (In *et al*., 2012). On the other hand, heat killed MIP was shown to inhibit NF-κB activation in melanoma cancer therapy (Halder *et al*., 2015). To evaluate the consistency of suppression effect of ACA and MIP on NF-κB in drug combination, western blot analysis was performed on NF-κB protein expression. As TNF-α is one of the main cytokines which binds to TNF receptors and can directly activate the NF-κB pathway, this study also sought to determine whether both double and triple combinations could suppress TNF-induced NF-κB activation.

MCF-7 breast cancer cells were treated with MIP/ACA, MIP/CDDP and MIP/ACA/CDDP at IC_{10} values. Cells were also pre-treated with TNF-α for 1 hr prior to treatment with double and triple combinations. Nuclear and cytoplasmic cell lysates were fractionated on 12 % (w/w) SDS-polyacrylamide gels, transferred into nitrocellulose membrane and analyzed using antibodies against NF-κB key protein members, p65 (detects free p65) and IκBα. The GAPDH protein, which is a housekeeping protein and constitutively expressed at high levels was used as a loading control for normalization purposes.

Analysis of NF-κB heterodimers translocation between the nucleus and cytoplasm using p65 revealed that levels of p65 in cytoplasm increased corresponding to increasing treatment time with MIP/ACA, MIP/CDDP and MIP/ACA/CDDP (Figure 4.9). This was consistent with a reduction in nuclear p65 levels, indicating that p65 was being shuttled out from the nucleus at a faster rate compared to its translocation rate into the nucleus, which indicated NF-κB inhibitory mechanism existed for all double and triple combinations in MCF-7. In addition to that, the ability of all three agents/drugs, to quench TNF-α based activation of NF-κB was also assessed. Results indicated MIP, ACA and CDDP in combinations were able to diminish TNF-α based NF-κB activation through the reduction in p65 levels in both cytoplasm and nuclear regions. This suggested that all the agents/drugs, could hypothetically induce apoptosis in various TNF-α transformed malignancies in addition to MCF-7 breast adenocarcinoma.

4.6.3 The effect of MIP, ACA and CDDP combinations on IκBα

NF-κB is a p50/p65 protein complex which is located within the cytoplasm and complexed to its inhibitor, IκBα. The role of inhibitor is to prevent nuclear translocation, thus suppress NF-κB activation. Phosphorylation and proteolysis of IκBα activates NF-κB p65 translocation into the nucleus. Western blotting analysis were conducted using antibodies against total IκBα and phosphorylated form of IκBα proteins, which is a marker required for ubiquitination signalling. However, Figure 4.9 indicated, IκBα levels were reduced upon double and triple combination treatment while phosphorylation level of IκBα proteins were increased. This led to the suggestion that MIP, ACA and CDDP in combination does not prevent the degradation of IκBα thus, IKK based phosphorylation and subsequent ubiquitination signalling will occur which will result in NF-κB activation. However, the reduction is not significant. The level of IκBα protein expresison was not quenched in MCF-7 cells, possibly due to the presense of other forms of NF-κB

heterodimers, such as, p52/RelB or p52/c-Rel which hypothetically countered the quenching of RelA/p50 based inhibition of IκBα expression. In TNF-α pretreated cells, IκBα proteins were not detected while its phosphorylated form was only present at 3 hr post treatmet. NF-κB is highly context-dependent and its activation patterns and their outcome differ from stimulus to stimulus and from cell-type to cell-type (Shih *et al*., 2011).

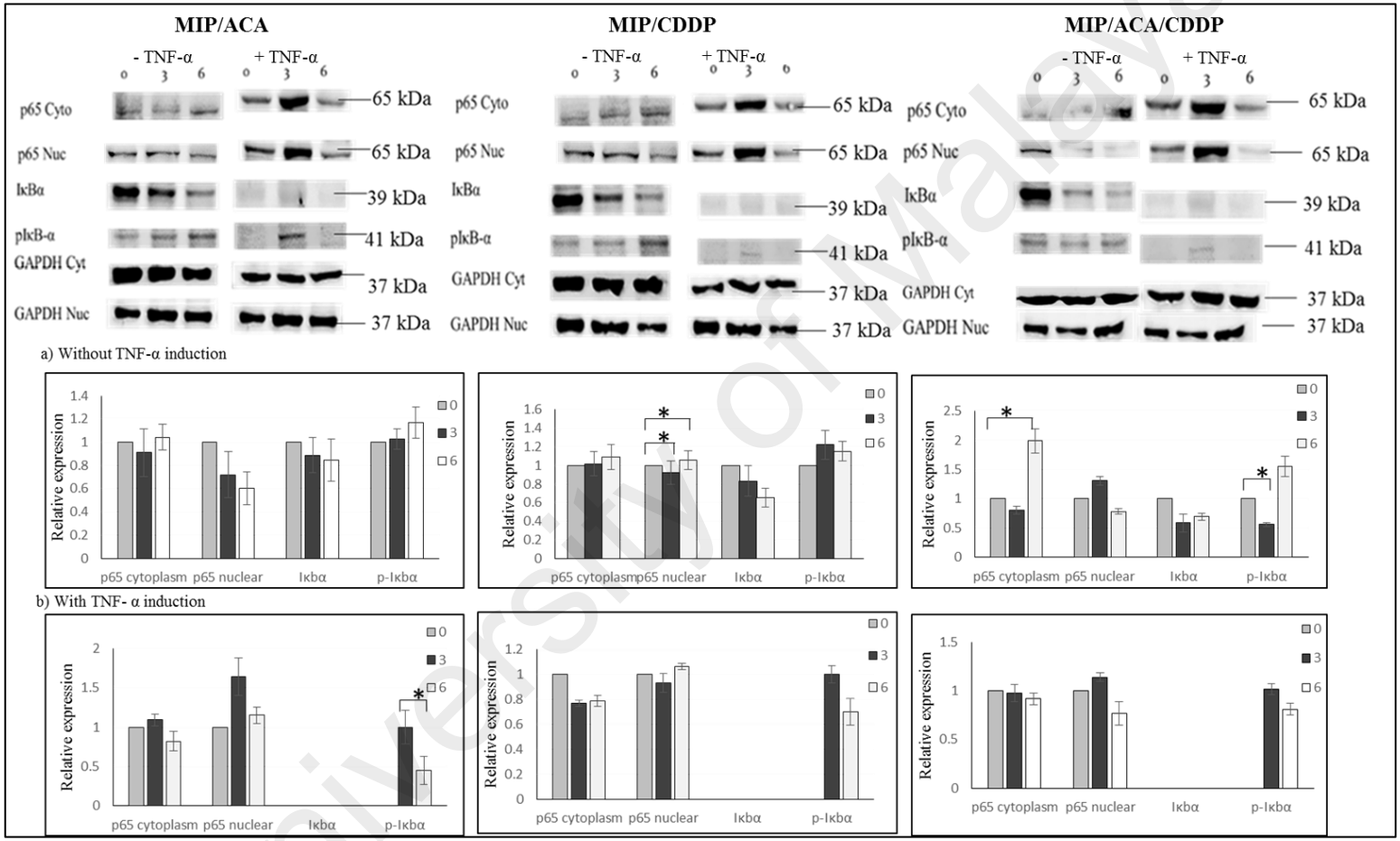


Figure 4.9: Combinations involving MIP, ACA and CDDP reduced NF-κB activation and inhibited p65 (RelA) nuclear retention in MCF-7 human breast cancer cells. Cells were treated with MIP/ACA, MIP/CDDP and MIP/ACA/CDDP at 3 and 6 hr in the presence and absence of TNF-α. (a) Normalized quantification of NF-κB protein members on double and triple treatments. (b) Normalized quantification of NF-κB protein members on TNF-α stimulation followed by double and triple treatments. All band intensities were quantified and normalized against GAPDH using the ImageJ v1.43u software, and presented as mean ± SEM of three replicates.

4.7 *In vivo* animal model

In vitro analysis of MIP, ACA and CDDP showed their potential cytotoxicity effect against various cancer types both in standalone and in combination analysis. Synergistic interaction of MIP/ACA, MIP/CDDP and MIP/ACA/CDDP was identified in breast cancer cell line, MCF-7. Mechanism of cell death upon single and combination treatments was proven to be induced via apoptotic cell death and dysregulated NF-κB activation.

Animal model have been used as the front line in predicting efficacy and finding toxicities for cancer chemotherapeutic agents before entering clinical trial. Thus, to investigate whether the results observed *in vitro* could also be seen *in vivo*, animal model studies were conducted using BALB/c mice treated with standalones and various combination regimens: MIP, ACA, CDDP, MIP/ACA, MIP/CDDP and MIP/ACA/CDDP. Since MIP is an immune-potentiator, a mice model with active immune system (BALB/c) was selected. Breast cancer was induced in BALB/c with allografted 4T1 mouse breast tumour cells, injected subcutaneously in the mammary fat pad. Treatment was started in seven groups with 6 mice in each group once the tumour was seen.

This animal model study was conducted to observe the effects on changes in tumour volume, assessment of body weight and monitoring of physiological side effects. Milliplex ELISA was performed to determine cytokines level while IHC assay was conducted on tumour biopsies to verify systemic drug effects. Hematoxylin and eosin (H&E) staining was performed on major organs to analyze toxicity level upon treatment.

4.7.1 Physiological effects of MIP, ACA and CDDP on BALB/c

Figure 4.10 represented the *in vivo* effects of treatment after 35 days post implantation and 30 days post-treatment with various ACA/MIP/CDDP treatment regimens and the orthotopic allograft tumour were harvested and photographed. Figure 4.11 showed harvested tumour and major organs like lung, heart, spleen and kidney from all treatment groups and healthy mice.

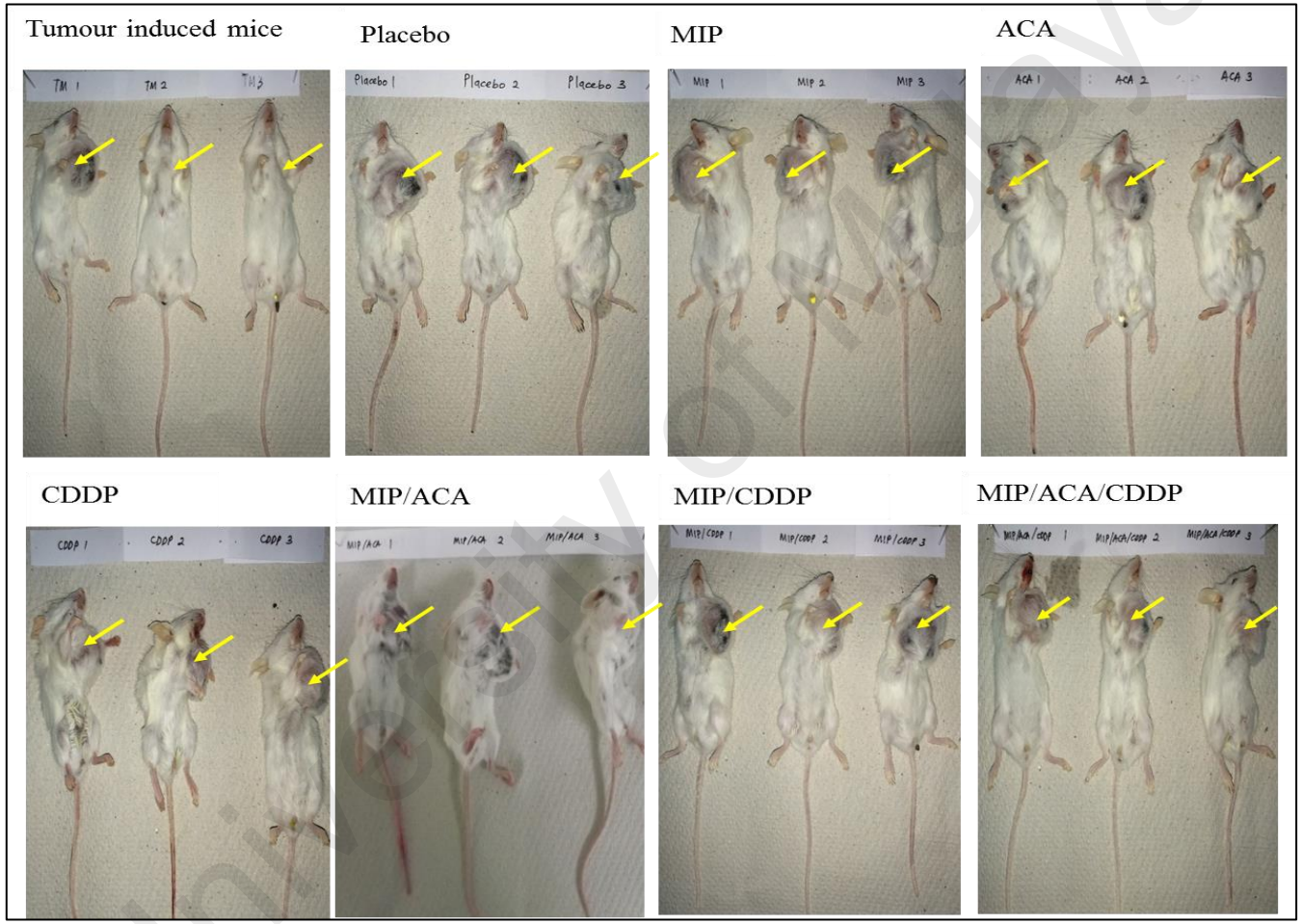


Figure 4.10: Photographs of BALB/c mice harvested 42 days post-implantation with mouse breast cancer 4T1 and 35 days post-treatment with various MIP, ACA, and CDDP treatment regimens. All mice were terminated via euthanasia, using a flow of pure CO_2 in a gas chamber. Dissections were carried out, and tumours were measured and fixed in 10 % (v/v) NBF buffer solution for IHC analysis. Locations of tumour sites were indicated by arrows.

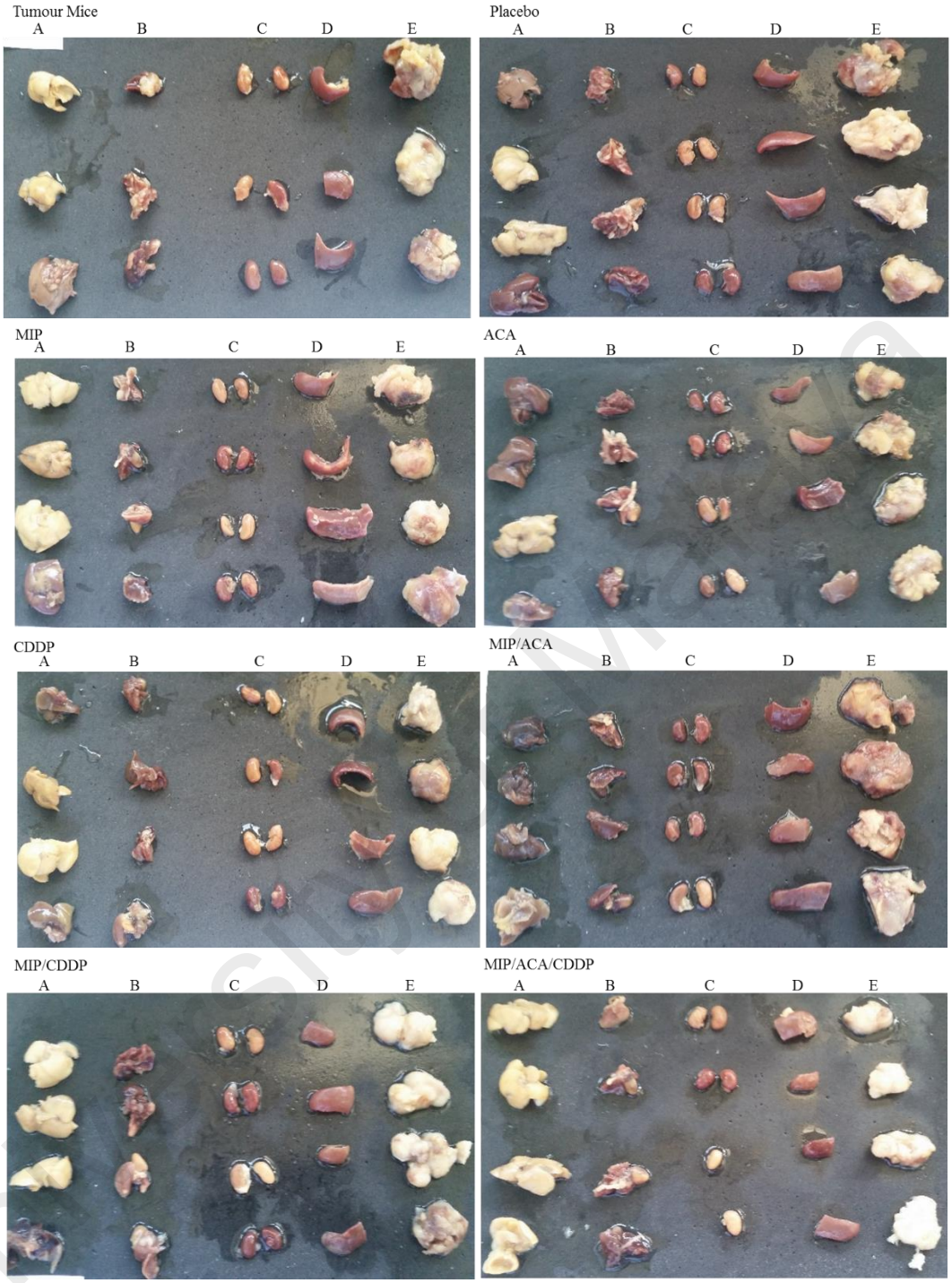


Figure 4.11: Photographs of major organs and tumour harvested 42 days post-implantation with mouse breast cancer 4T1 and 35 days post-treatment with various MIP, ACA, and CDDP treatment regimens. A) Lung; B) Heart; C) Kidney; D) Spleen; E) Tumour. All mice were terminated via euthanasia, using a flow of pure CO_2 in a gas chamber. Dissections were carried out, and tumours were measured and fixed in 10 % (v/v) NBF buffer solution for IHC analysis.

4.7.2 Toxicity evaluation of the organs

Toxicity to the major organs was evaluated using hematoxylin and eosin staining of their paraffin sections. Obvious damage in lungs and mild damage to the other major organs, such as heart, liver, spleen and kidney was observed at the end of treatments. However, the toxicity of major organs that was observed in treatment groups are due to the side effects of 4T1 breast cancer and are not related to treatments since tumour induced mice (without treatment) showed similar toxicity effects. Moreover, 4T1 tumour is highly tumourigenic and invasive, unlike most tumour models, can spontaneously metastasize from the primary tumour in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone (Pulaski & Ostrand-Rosenberg, 1998; Lelekakis *et al*., 1999). The progressive spread of 4T1 metastases is very similar to that of human breast cancer (ATCC). Morphology of the tumour and major organ tissues from the seven treatment groups was shown in Figure 4.12.

In 4T1 tumour induced mice group (placebo), breast tumour metastasis was observed in heart, liver and lung while kidney shows normal morphology. In liver, acute inflammatory cells were present in lobules, portal tracts and were detected around the central veins as well.

In MIP treated group, H&E analysis revealed the absence of tumour cells development in heart, kidney, liver and spleen sectioning. However, acute myocarditis in heart, acute nephritis in kidney while acute hepatitis in liver was reported. Severe acute pneumonia with abscess formation foci of tumour metastasis was seen in lung sections. In spleen, tumour cells were not seen, however, hypoplasia of white with red pulp and filled with bizarre cells were present. Tumour was around 1.5 cm with about 50 % necrosis and poorly differentiated carcinoma without gland formation was reported.

In the second standalone drug treatment group (ACA), normal architecture of heart and liver were observed. In spleen, severe hyperplasia was reported but no definite tumour cells were detected. As predicted, tumour metastasis was seen in lung sectioning. Increased inflammation was also seen in the kidneys. In the harvested tumour, approximately 50 % of necrosis was achieved with no obvious squamous or granular differentiation.

Kidney and heart showed morphologically within normal limit in CDDP treated group mice. Liver was generally in normal architecture. Hepatocytes and portal tracts with only focal lobular hepatitis were noticed. In lungs, tumour metastasis was seen only focally and surrounding cells were still with normal-looking alveoli with interstitial pneumonitis. Hyperplasticity in white and red pulp in spleen with scattered bizarre looking giant cells indicating tumour infiltration has occurred. The tumour was presented with 80 % necrosis without obvious squamous.

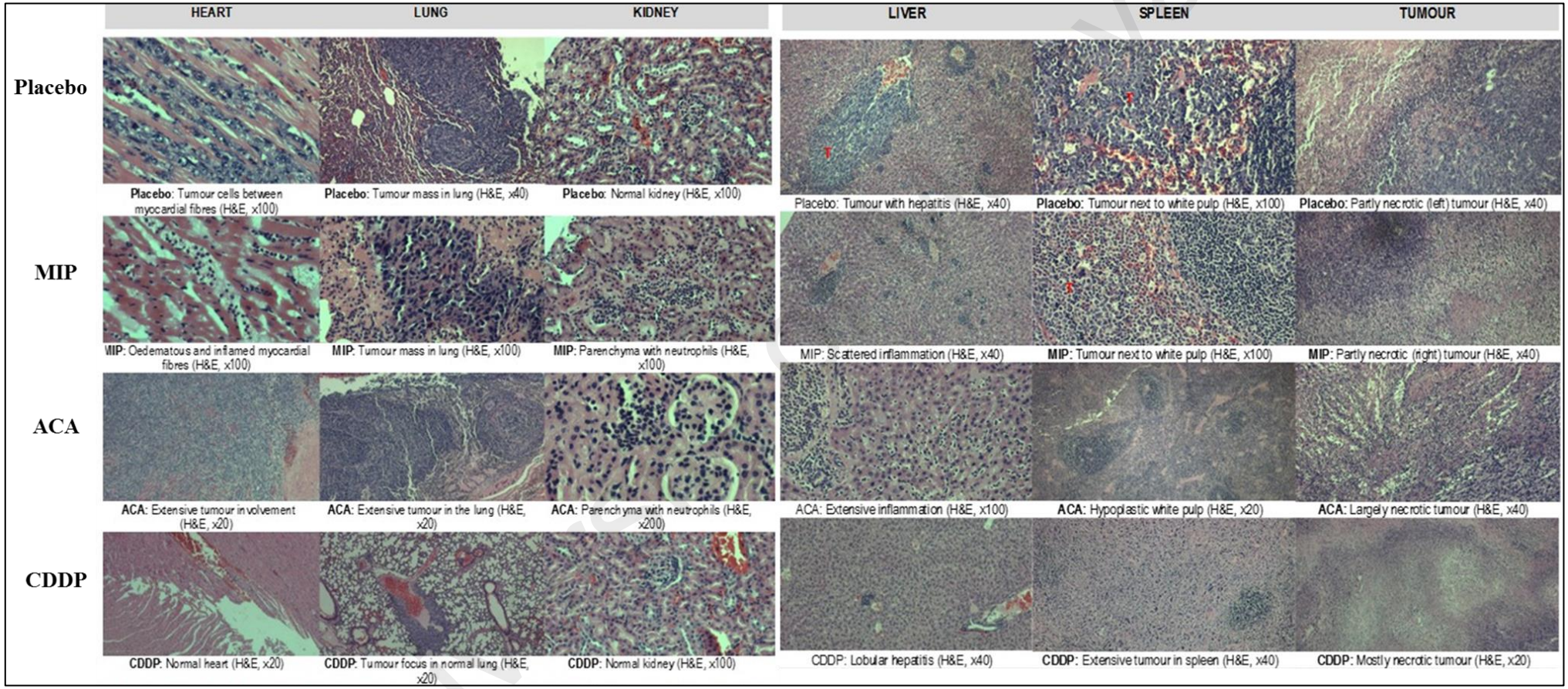
In double combination of MIP/ACA treatment group, definite tumour was not detected in heart, liver, kidney and spleen. However, minor destructions were seen, such as, severe hypoplasia of white and red pulp sinusoids in spleen, liver sinusoids and acute inflammation in the myocardium was noted. In lung, tumour nodules were presented with adjacent area showing oedema and interstitial pneumonitis. Tumour marked 80 % necrosis with poorly differentiated carcinoma without gland formation.

In MIP/CDDP treated group, heart and kidney were morphologically within normal limit. Only mild focal lobular hepatitis were seen in liver with occasional small tumour clusters were detected. Tumour metastasis was also detected in lung while mild hypoplasia was

seen in the spleen. Tumour sections revealed approximately 50 % necrosis with poorly differentiated carcinoma formation.

In triple drug treated groups, as expected tumour metastasis were detected in the lungs. In the spleens, hypoplasia of white and red pulp sinusoid was reported, and it was filled with tumour cells while liver, heart and kidney were within limit. The tumour harvested from this group had almost 100 % achieved necrosis with poorly differentiated carcinoma with no gland formation seen.

Compared to the placebo group, mice treated with combination drugs exhibited features associated with necrosis and infiltration of inflammatory cells in their tumour tissues which is an indication of anti-tumour activity of the combination drugs.


HEART
LUNG
KIDNEY
LIVER
SPLEEN
TUMOUR
Placebo
MIP
ACA
CDDP
Placebo: Tumour cells between myocardial fibres (H&E, x100)
Placebo: Tumour mass in lung (H&E, x40)
Placebo: Normal kidney (H&E, x100)
Placebo: Tumour with hepatitis (H&E, x40)
Placebo: Tumour next to white pulp (H&E, x100)
Placebo: Partly necrotic (left) tumour (H&E, x40)
MIP: Oedematous and inflamed myocardial fibres (H&E, x100)
MIP: Tumour mass in lung (H&E, x100)
MIP: Parenchyma with neutrophils (H&E, x100)
MIP: Scattered inflammation (H&E, x40)
MIP: Tumour next to white pulp (H&E, x100)
MIP: Partly necrotic (right) tumour (H&E, x40)
ACA: Extensive tumour involvement (H&E, x20)
ACA: Extensive tumour in the lung (H&E, x20)
ACA: Parenchyma with neutrophils (H&E, x200)
ACA: Extensive inflammation (H&E, x100)
ACA: Hypoplastic white pulp (H&E, x20)
ACA: Largely necrotic tumour (H&E, x40)
CDDP: Normal heart (H&E, x20)
CDDP: Tumour focus in normal lung (H&E, x20)
CDDP: Normal kidney (H&E, x100)
CDDP: Lobular hepatitis (H&E, x40)
CDDP: Extensive tumour in spleen (H&E, x40)
CDDP: Mostly necrotic tumour (H&E, x20)

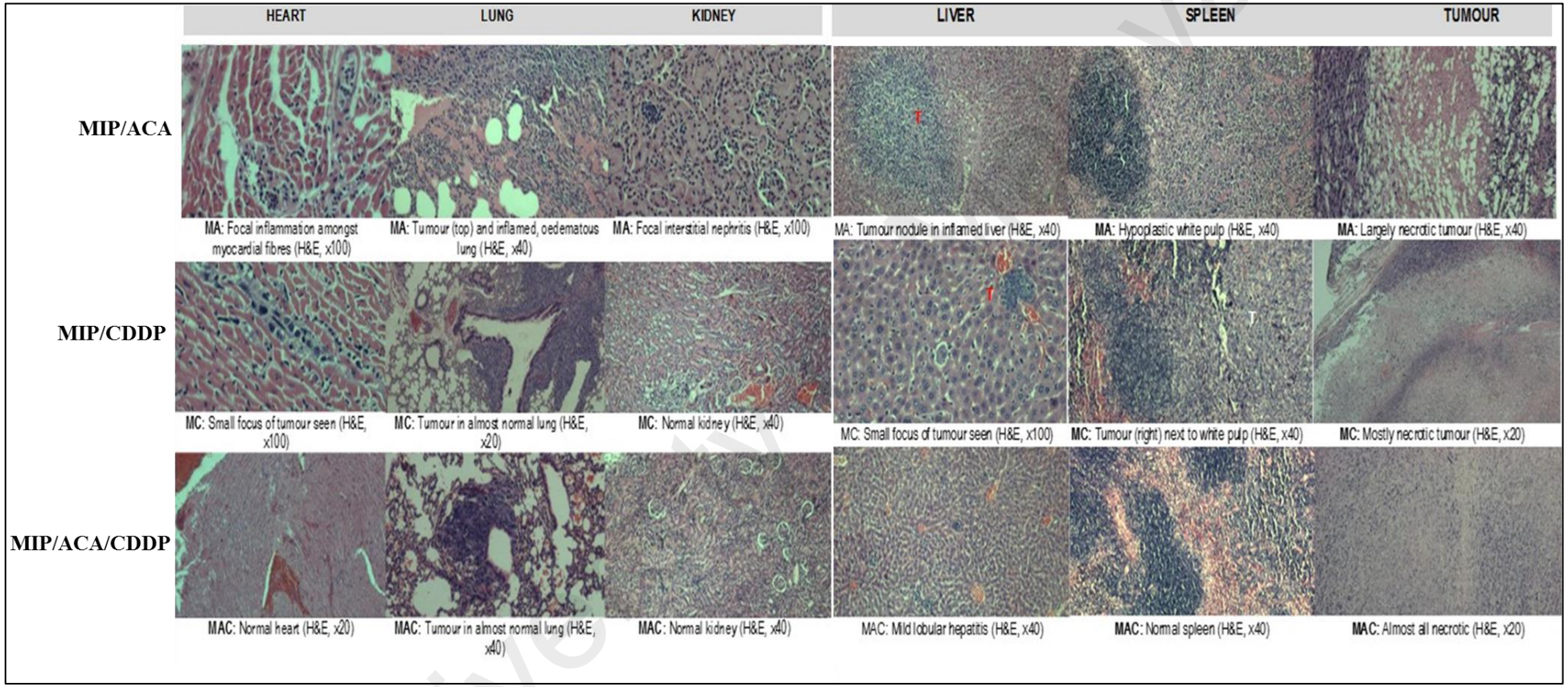


Figure 4.12: Preliminary toxicity evaluations in the hearts, lungs, kidneys livers, spleens and tumours (**T**) bearing BALB/c mice after treatment with saline, MIP, ACA and CDDP as standalones and in combinations. Paraffin sections of major organ tissues were stained with hematoxylin and eosin. Images were obtained under a microscope at 400× magnification.

4.7.3 Tumour volume & body weight

Next, to determine the effect of various treatments on mice models, tumour volume and body size was observed throughout tumour induction and treatment period. The result in Figure 4.13 demonstrated the impact of standalone and drug combination treatment on the mean tumour volume while the body weight for each treatment groups are presented in Figure 4.14.

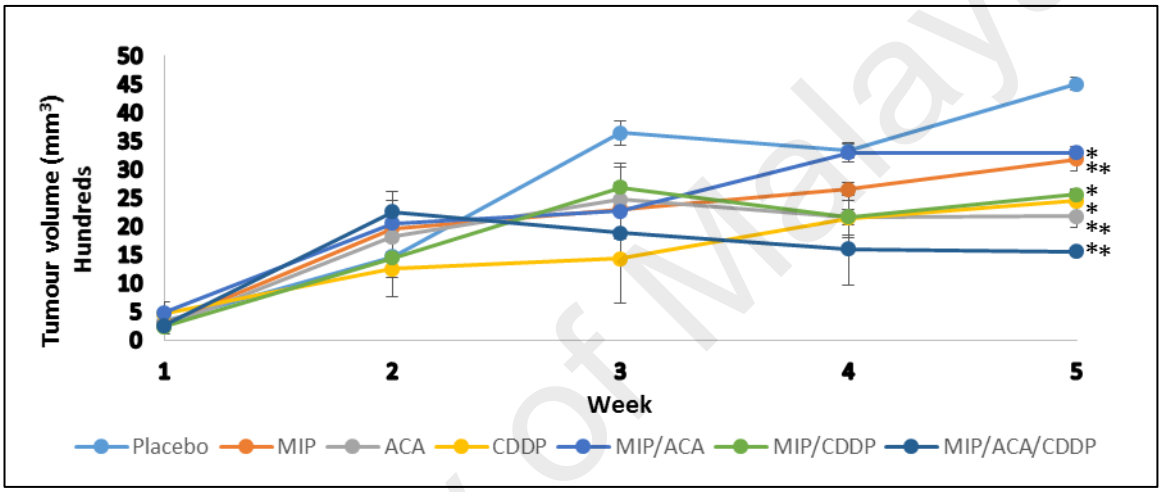


Figure 4.13: Tumour growth curve of tumour-bearing mice injected with different regimens over a period of 5 weeks. Tumour volume was calculated from second week. Data were shown as mean ± SD.

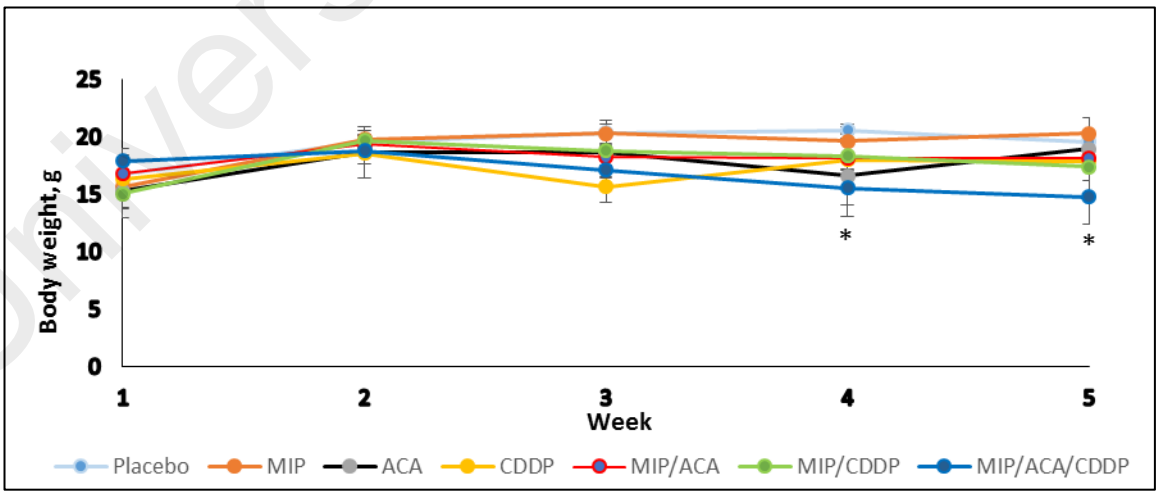


Figure 4.14: Body weight change in 4T1-bearing mice treated with different regimens. The values were shown as mean ± SD.

The mice treated with MIP/ACA/CDDP showed greater tumour volume reduction of about 65 % compared to placebo. Second highest tumour volume reduction was observed in ACA treated group (46 %) followed by CDDP (46 %), MIP/CDDP (43 %), MIP (29 %) and the least reduction seen in MIP/ACA (27 %) treated group.

Body weights of mice was maintained at 15-20 g except in triple drug combination group with body weight reduction marked around 25 % compared to placebo.

4.7.4 Immunohistochemistry

In previous *in vitro* analysis, MIP, ACA and CDDP in standalones and combinations, was shown to mediate their anti-cancer effects through the NF-κB signaling pathway. Since NF-κB is one of the vital regulators of pro-inflammatory gene expression, it induces the transcription of a wide variety of inflammatory-related elements, such as, pro-inflammatory biomarkers, cytokines, chemokines, adhesion molecules, growth factors and apoptotic genes (Ghosh *et al*., 1998; Tak & Firestein, 2001).

Immunohistochemistry analysis was carried out to determine expression of NF-κB regulated genes (p65 & pIKKα/β) and inflammatory biomarkers, such as, cleaved caspase-3 (CC-3), cyclin D1 (CD1), matrix metallopeptidase-9 (MMP-9), histone deacetylase (HDAC), histone acetyltransferase p300, cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor p21, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2) on standalone and drug combination treated and placebo 4T1 allograft tumour biopsies. Figure 4.15 showed the quantification of the relative intensity of DAB staining in all the IHC analysis on 4T1 tumour sections.

4.7.4.1 Effects of standalone and combination treatment on NF-κB regulated genes

Figure 4.16 depicted IHC staining for p65 (RelA), an NF-κB subunit on 4T1 tumour tissue. This protein was chosen because p50/p65 complex has been shown to be most common and dominant NF-κB heterodimer form in most cancer types (Loercher *et al*., 2004). The expression of protein level found to be higher in tumour tissue in comparison with placebo sections (70.66±4.9) followed by MIP (69.59±3.0) and MIP/CDDP (69.4±1.8) treated sections. Lower expression was found in the sections from ACA treated mice with mean intensity of 64.3±3.4. CDDP (66.4±4.6) and MIP/ACA (67.2±0.6) treated mice show moderate p65 expression. This results showed, p65 a key protein of NF-κB, inhibited in all treatment groups, and therefore inactivation of this pathway has occurred during the treatments. Moreover, pIKKα/β analysis (Figure 4.17) revealed decreased expression in most of the treatment except in MIP/CDDP (97.09±1.1) and MIP/ACA/CDDP (95.1±10.1) treated tumour sections.

4.7.4.2 Effects of standalone and combination treatment on inflammatory biomarkers

To further investigate the role of apoptosis, tumour sections were stained with CC-3 antibody, an executioner enzyme of apoptosis. CC-3 is a cleaved fragment of caspase 3. CC-3 can detect cleaved and active caspase-3 in dying cells. After proteolytic cleavage between Asp 175 and Ser 176 which separated large and small subunits leads to the activation of caspase-9, therefore the epitope is exposed and can be easily detected by CC-3 (Fan & Bergmann, 2010).

A slight increase in expression of CC-3 was seen in tumour sections of double and triple combination treated mice as compared to placebo. The expression was reduced in single

drug treated groups as shown in Figure 4.18. The highest expression was obtained in MIP/CDDP (114.9±3.2) followed by MIP/ACA (110.8±3.1) and MIP/ACA/CDDP (110.4±2.0). This result gave an insight that high level of apoptosis was achieved with combination treatment compared to single drug treatments. Moreover, it was also consistent with tumour regression as shown in Figure 4.13, as well as the outcome of the H&E analysis where higher necrotic cell were observed in combination treatments as compared to single agent treatments since apoptosis can leads to secondary necrosis (Silva, 2010).

CD1 is a proto-oncogene involves in cell cycle progression. It regulates progression of G1 to S phase in many different cell types. CD1 has been linked to the development and progression of breast (Gillet *et al*., 1996), esophagus, bladder and lung cancers (Knudsen *et al*., 2006). The overexpression of this protein occurs mainly due to the consequence of gene amplification and also its defective regulation at the post-translational level (Gillett *et al*., 1994; Russell *et al*., 1999). During overexpression, G1 phase shortens and results in less dependency on growth factor thus, abnormal cell proliferation occurs which in turn favours the occurrence of additional genetic lesions (Todd *et al*., 2002).

In this study, increased expression of CD1 (Figure 4.19) has been observed in MIP (121.8±4.2), MIP/ACA (123.8±7.7), MIP/CDDP (127.2±2.5) and MIP/ACA/CDDP (121±2.2) treated groups compared to placebo (120±2.3) while a decrease in CD1 expression level was achieved in ACA (108.4±1.6) and CDDP (110±5.2) treated groups. These results clearly highlight the involvement of MIP in inducing CD1 expression and consequently result in abnormal cancer cell proliferation.

CD1 forms active complex with its binding partner CDK4 to promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein, pRb (Kato *et al*., 1993). Therefore, CDK4 as well as play a key role in cell proliferation, where its overexpression induces proliferations and development of cancer. Decreased CDK4 expression was only seen in MIP/ACA and CDDP treated group while the protein expression was slightly increased in other treatment groups as shown in Figure 4.20.

MMP-9 is a member of matrix degrading enzyme involved in cancer development, invasion and metastasis. Differential expression of MMP-9 reflects the extent of cellular differentiation in breast cancer and is closely related to the most aggressive subtype of breast cancer (Yousef *et al*., 2014). Its basal expression is usually low whereas it is highly expressed in most human cancer in response to various growth factors and cytokines (Mook *et al*., 2004). As shown in Figure 4.21, a prominent reduction in MMP-9 expression was seen in ACA (87.4±3.8) treated mice compared to the placebo group (137.4±4.9). Second highest reduction was seen in MIP/CDDP treated mice (102±8.6), followed by CDDP (103±10), MIP (109.7±6.8), MIP/ACA (120±0.8) and finally in the MIP/ACA/CDDP treated group (132.3±7.6). Therefore, the single, double and triple combination treatments can be correlated with the reducing occurrence of metastasis.

HDAC family members cause DNA damage by forming inactive chromatin structure. Removal of acetyl group from lysine residues of histone increases the interaction between histone and DNA, thus eventually represses DNA transcription (Yang *et al*., 2001). Increased deacetylation of histones leads to cell proliferation, apoptosis, cell migration, and invasion via inactivation of tumour suppressor gene (Seo *et al*., 2014). In this study, a decrease in HDAC expression was observed only in MIP and ACA treated group compared to other single and combination treatment groups (Figure 4.22).

p300 is a lysine acetyltransferase that catalyzes the addition of acetyl group to lysine residues of histone (Gu & Roeder, 1997). It acts as a double edged sword for tumour growth depending on the cell types and signaling pathways. Several studies has shown that p300 overexpression leads to tumourigenesis and cancer progression (Yokomizo *et al*., 2011; Santer *et al*., 2011) while other studies reported p300 may suppressed tumour growth and inhibited cancer progression in breast, colorectal and pancreatic cancers (Gayther *et al*., 2000; Iyer *et al*., 2004). As shown in Figure 4.23, high expression of p300 was observed in MIP/ACA treated group (113.2±5), followed by ACA (100.3±3.5), MIP/CDDP (99±2.8) and CDDP (95.6±2.6). The expression of p300 in the triple combination group was in moderate level while the least expression was observed in MIP standalone group with a reading of 93.7 ±2.3.

COX-2 is an inducible form of COX group enzymes which involves in the conversion of arachidonia acid to prostaglandins. Subsequently, prostaglandins induce the activation and expression of aromatase (Zhao *et al*., 1996) which further converts androgen to estrogen. Lastly the estrogen stimulates cancer cell growth via activation of estrogen receptors and its target genes. COX-2 is often overexpressed in breast cancer and its metabolites may contribute to tumour viability, hyper-proliferative growth, invasion and metastasis (Williams *et al*., 1999; Witton *et al*., 2004). In this study, COX-2 expression was reduced in all the treatment groups except in MIP/CDDP and MIP/ACA/CDDP treated groups (Figure 4.24). A prominent reduction was observed in MIP/ACA treated mice (150.4±22.3) followed by MIP (153.3±4.4), ACA (157±5.2) and CDDP (157.2±3.4) treated groups.

VEGF is a protein involve in tumour angiogenesis (Hicklin & Ellis, 2005) by initiating the formation of immature vessels for vascular formation. The expression of this protein

on tumour cells stimulates tumour cell growth (Erovic *et al*., 2005) and may also decrease tumour apoptosis (Koide *et al*., 1999). As shown in Figure 4.25, a great reduction of VEGF expression was noticed in all the treatment groups compared to placebo (73.8±2.9). The highest reduction was achieved in CDDP (58.21±6.5) followed by MIP/CDDP (59.6±4.1), MIP (59.7±3.8) and MIP/ACA with a mean intensity of 62.3±2.7. The least reduction of VEGF was achieved in ACA treated tumour section with a mean intensity of 65.1±5.5.

Cancer develops when the balance between cell proliferation and cell death is disrupted which result in aberrant proliferation leading to tumour growth. The cyclin dependent kinase inhibitor p21 plays a key role in preventing tumour development and the induction of p21 may result in cell cycle arrest (Gartel & Tyner, 2002). As shown in Figure 4.26, the protein expressions of p21 were found high in ACA treated section with a mean intensity of 64.95±5.23 compared to placebo tumour sections (50.41±1.4). ACA in combination with MIP showed the second highest expression of about 63.03±3.7, followed by CDDP (56.0±5.9) and MIP/CDDP (56.4±4.8) treated group. The least expression was seen in the triple combination group with a mean intensity of 51.6±0.7.

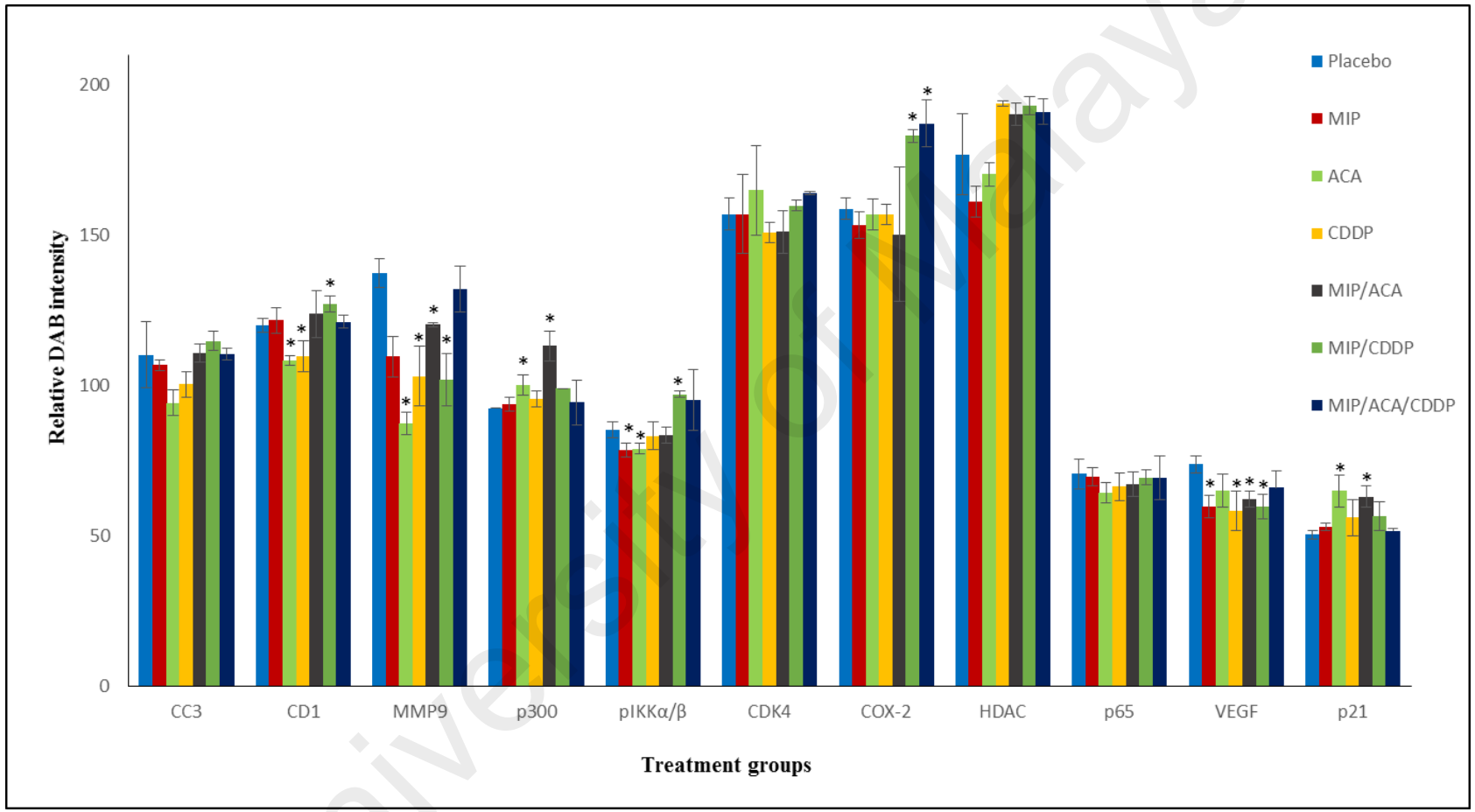


Figure 4.15: Quantification of relative intensity of IHC DAB staining on 4T1 breast tumour sections treated with various MIP, ACA and CDDP standalones, double and triple combinations. Data for all NF-κB regulated proteins and inflammatory biomarkers were presented as mean±SEM of three independent replicates.

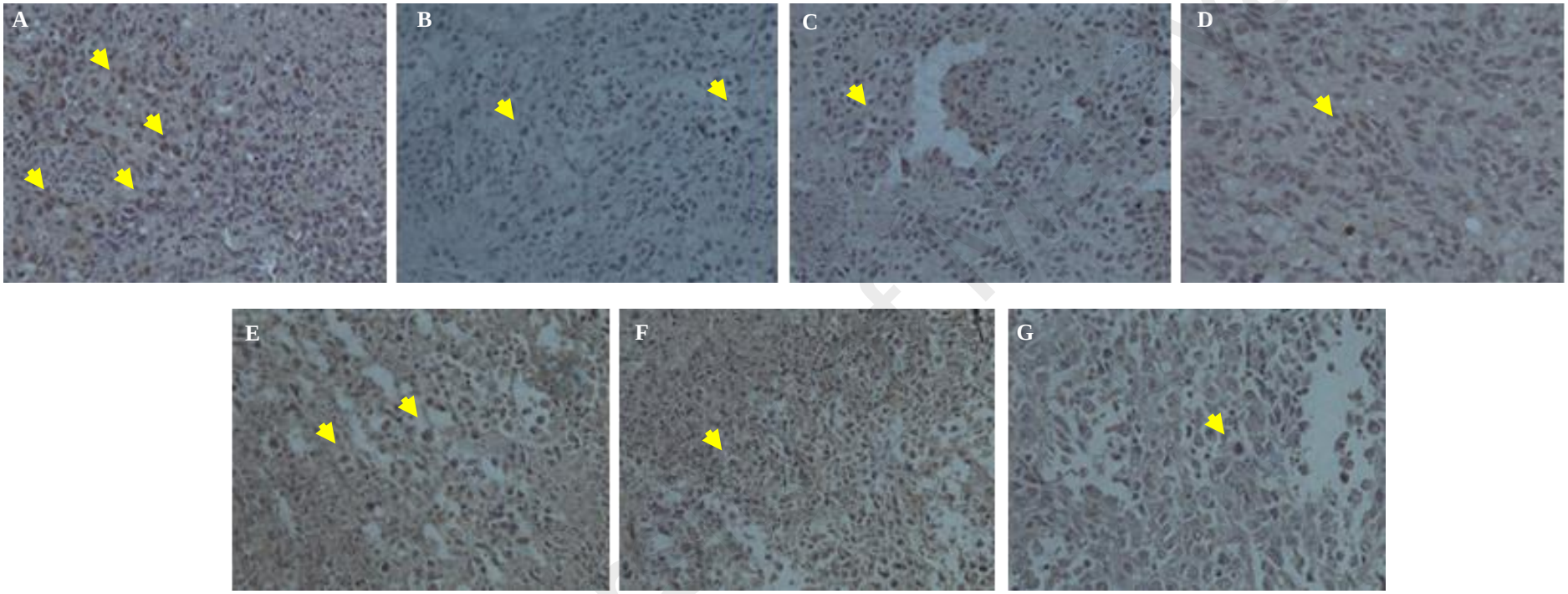


Figure 4.16: Immunohistochemical analysis of the expression of p65 in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

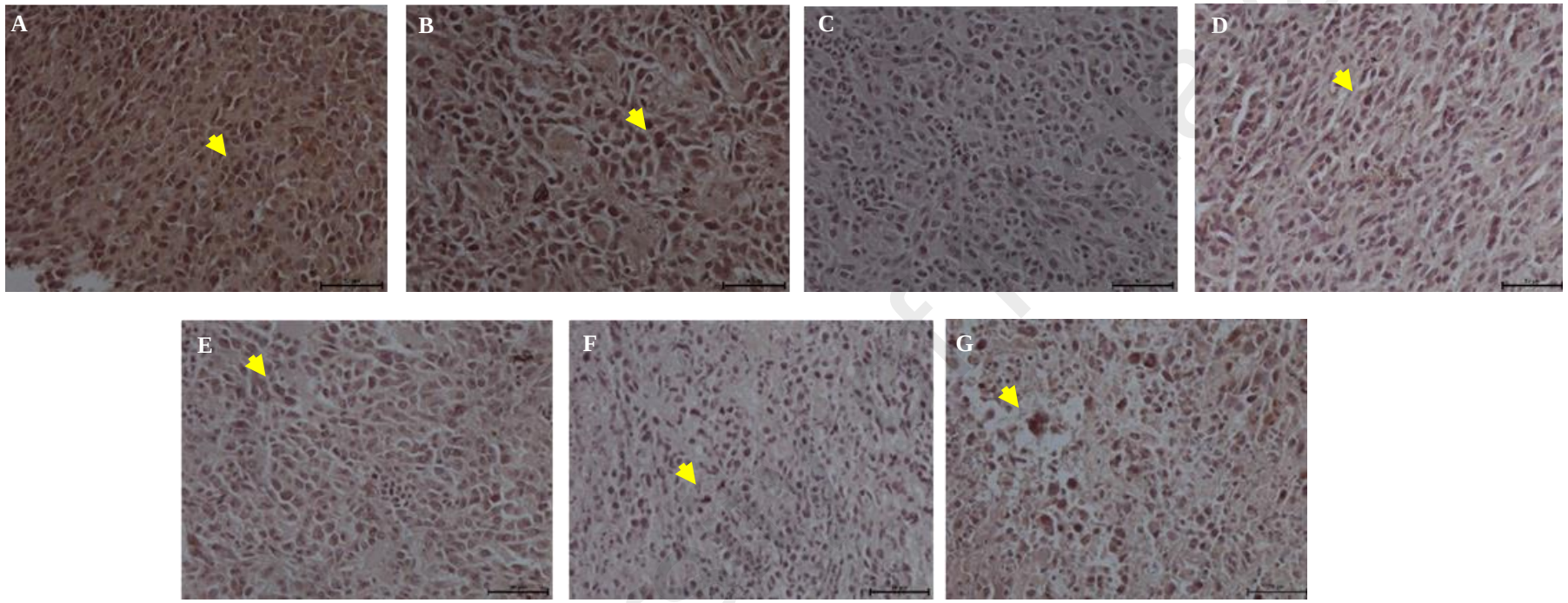


Figure 4.17: Immunohistochemical analysis of the expression of pIKKα/β in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

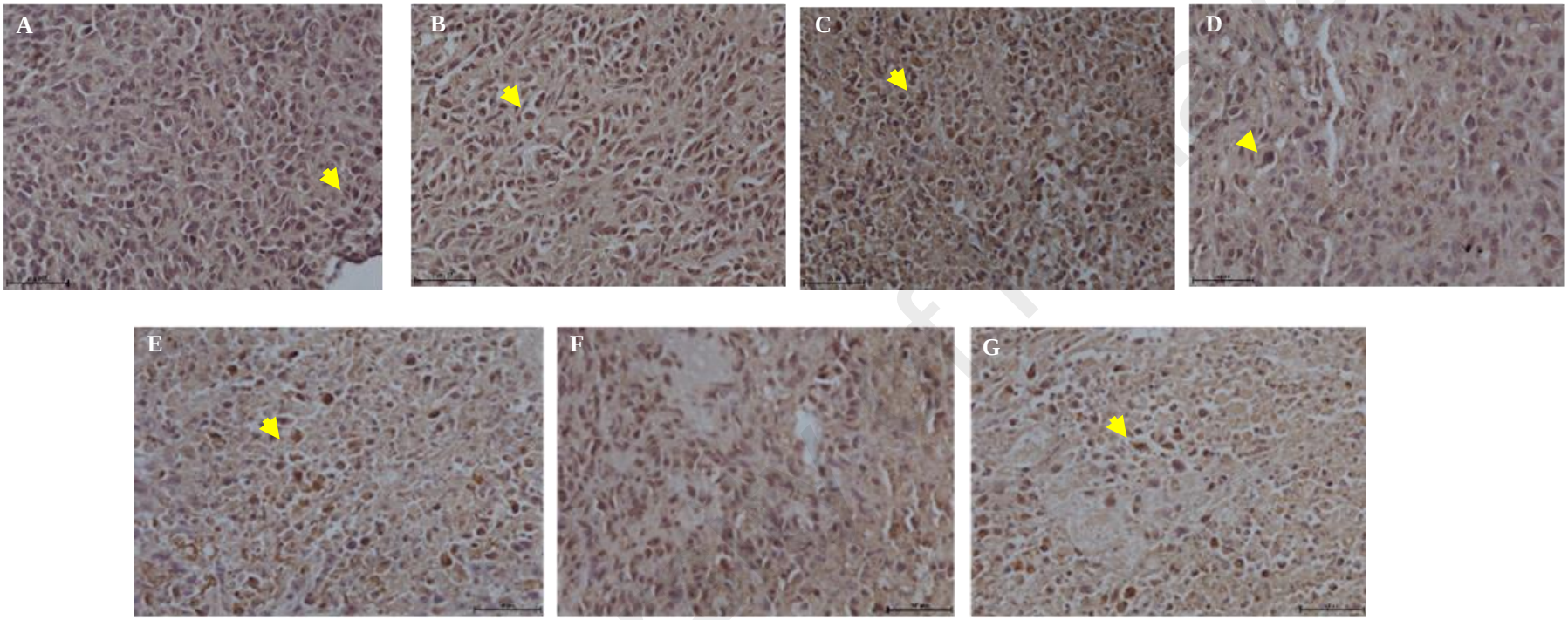


Figure 4.18: Immunohistochemical analysis of the expression of cleaved caspase-3 (CC-3) in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

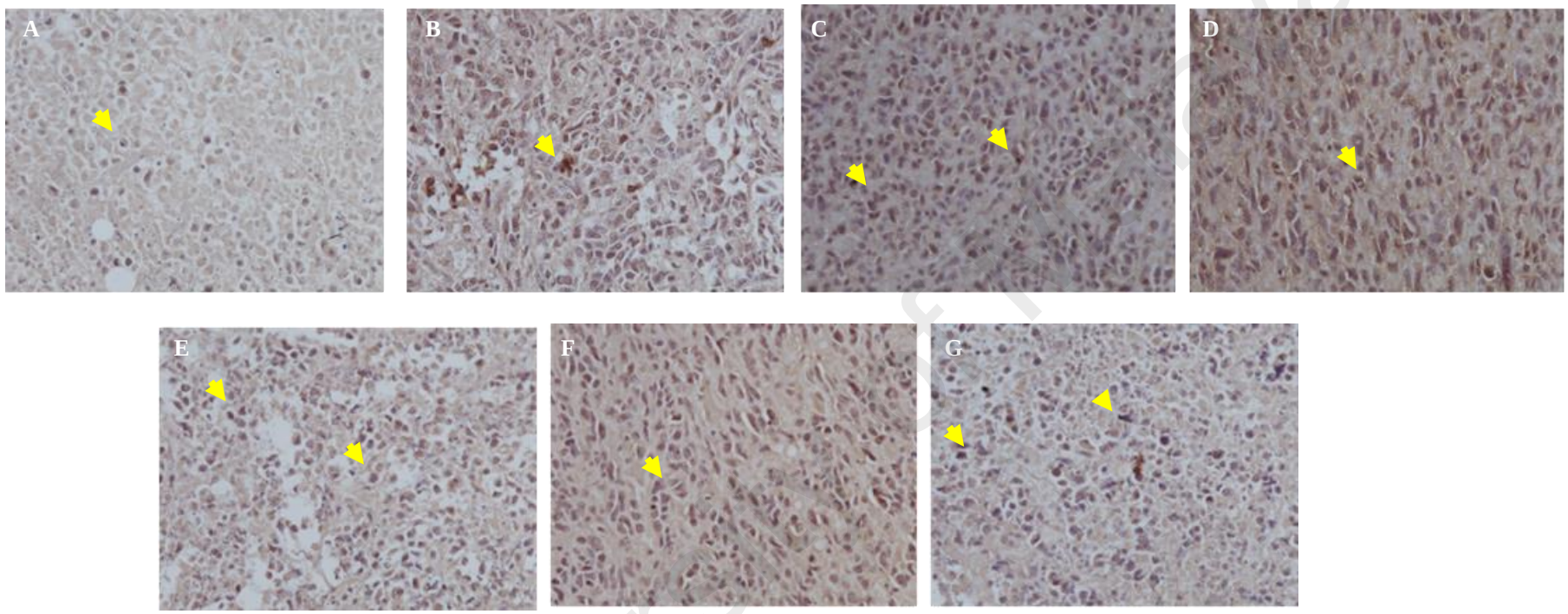


Figure 4.19: Immunohistochemical analysis of the expression of cyclin D1 (CD1) in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

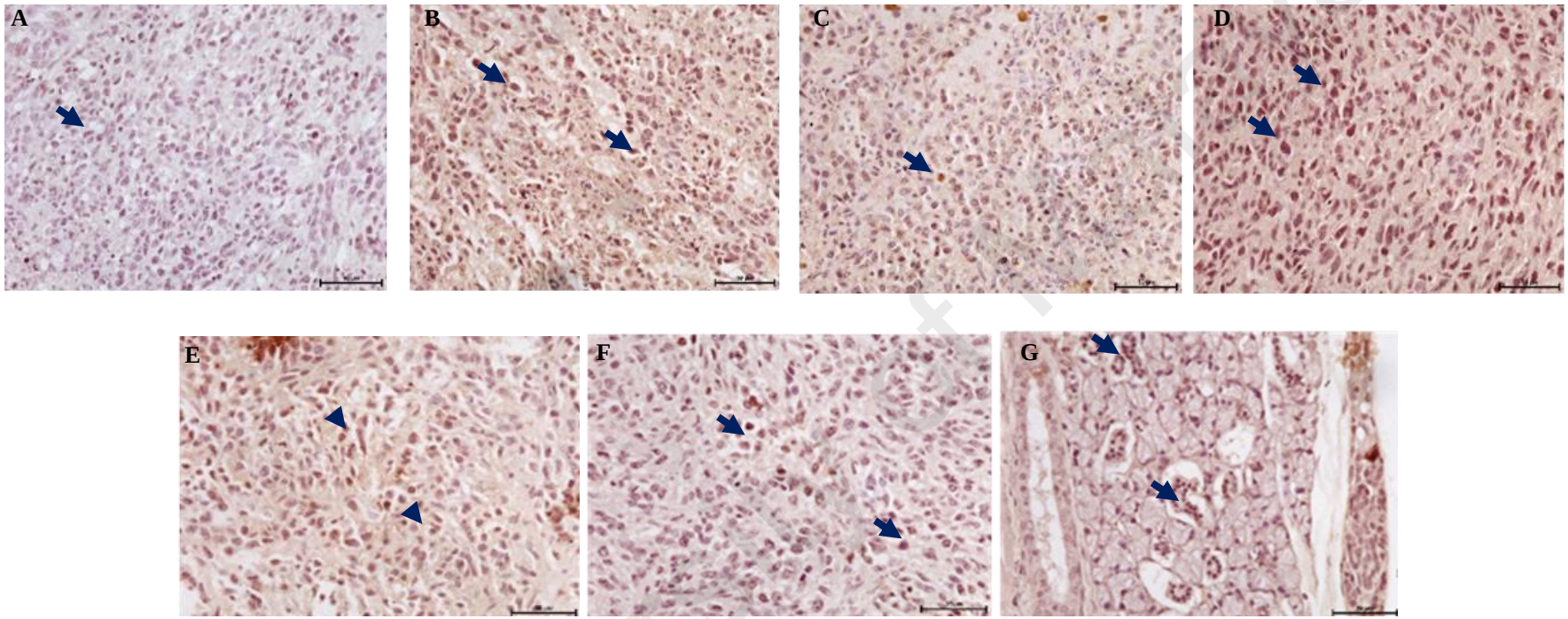


Figure 4.20: Immunohistochemical analysis of the expression of cyclin-dependent kinase 4 (CDK4) in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

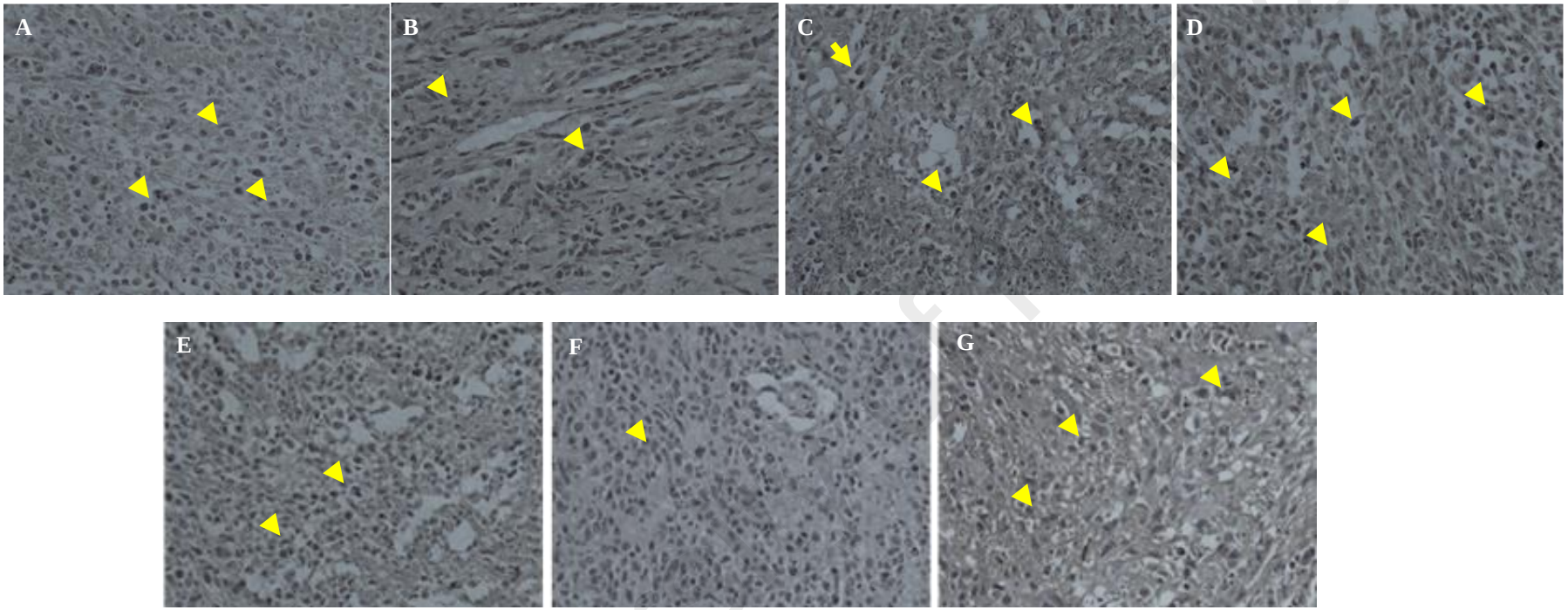


Figure 4.21: Immunohistochemical analysis of the expression of matrix metallopeptidase-9 (MMP-9) in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

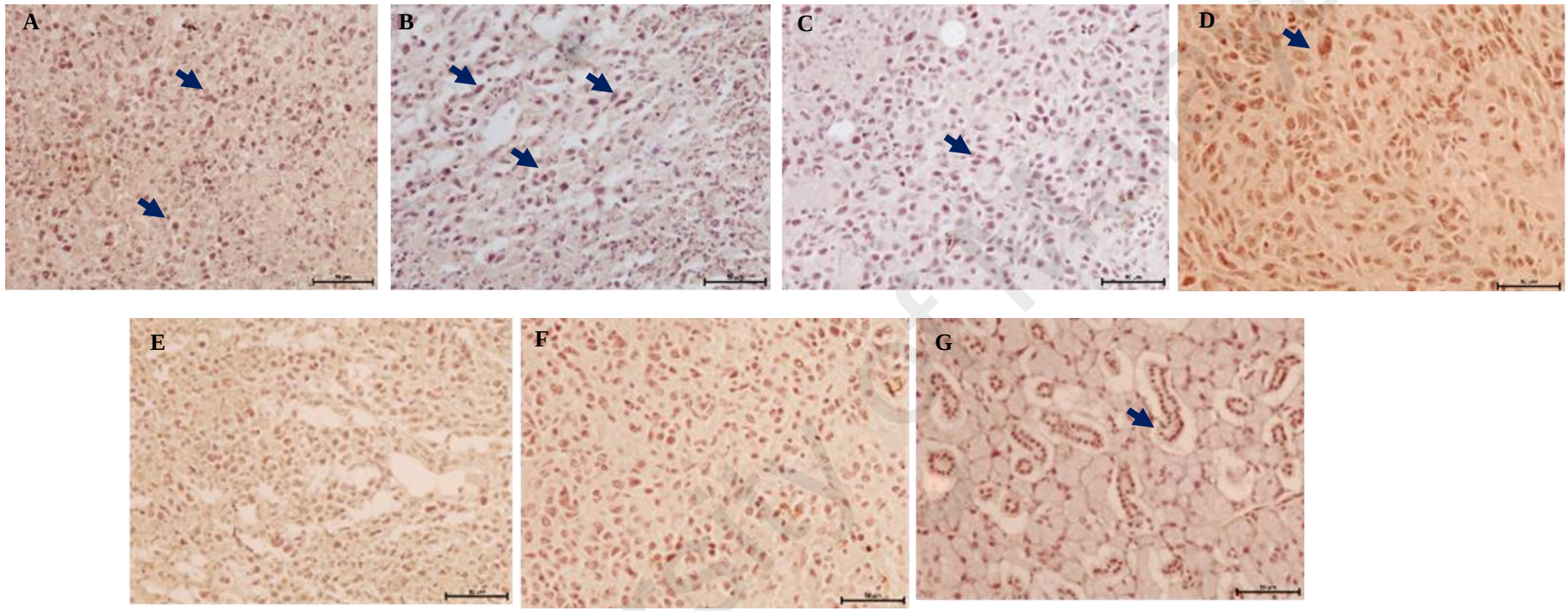


Figure 4.22: Immunohistochemical analysis of the expression of HDAC in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

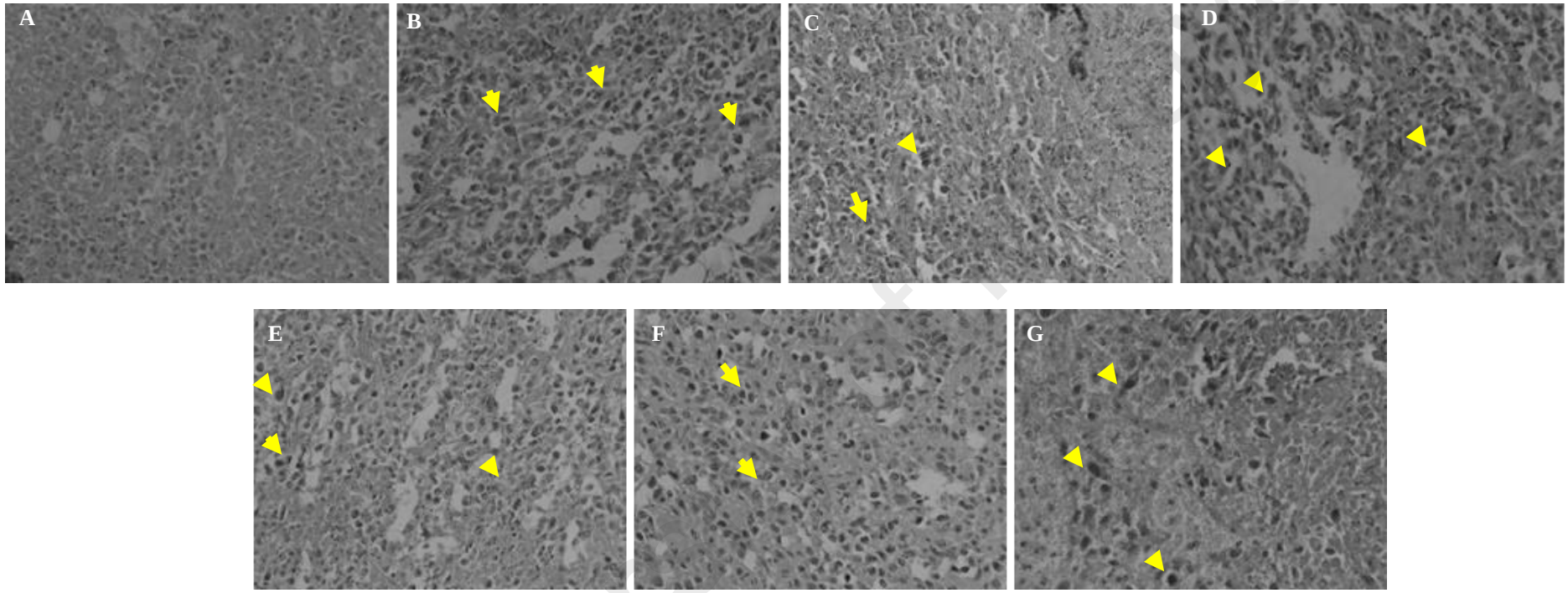


Figure 4.23: Immunohistochemical analysis of the expression of p300 in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

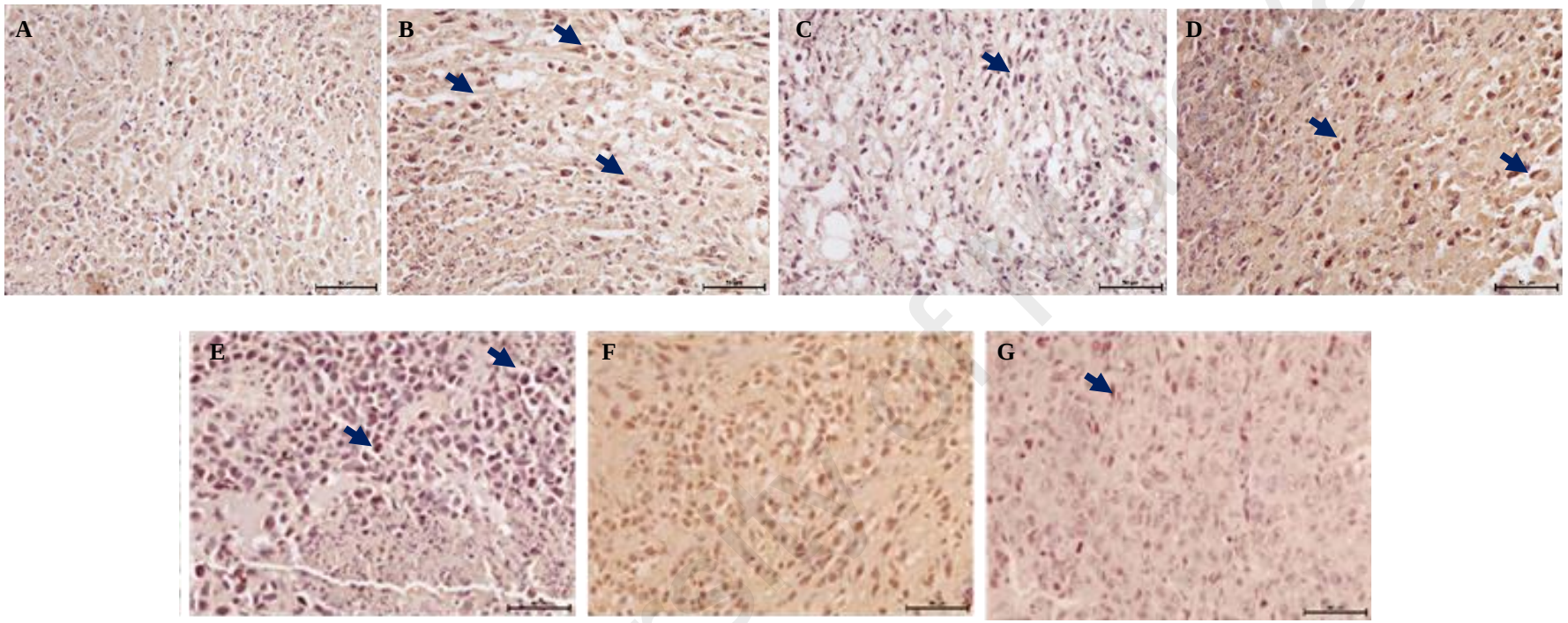


Figure 4.24: Immunohistochemical analysis of the expression of cyclooxygenase-2 (COX-2) in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

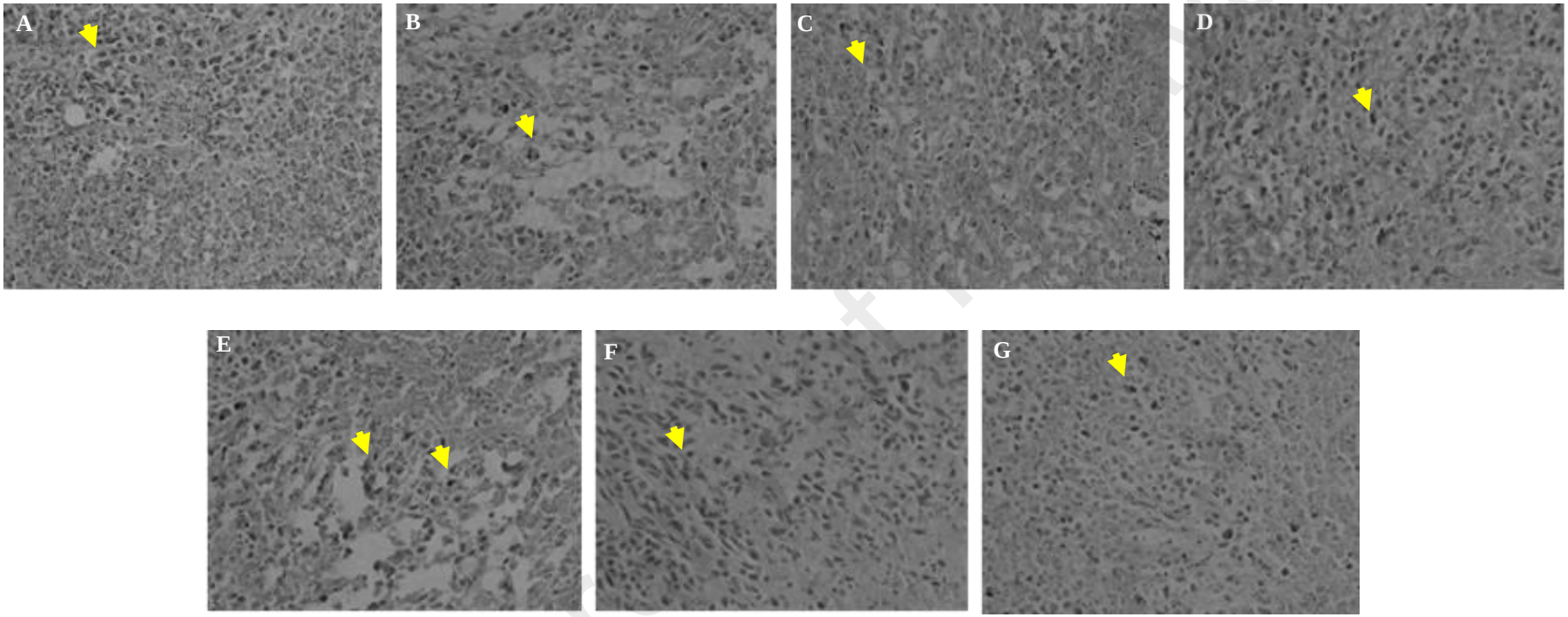


Figure 4.25: Immunohistochemical analysis of the expression of vascular endothelial growth factor (VEGF) in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

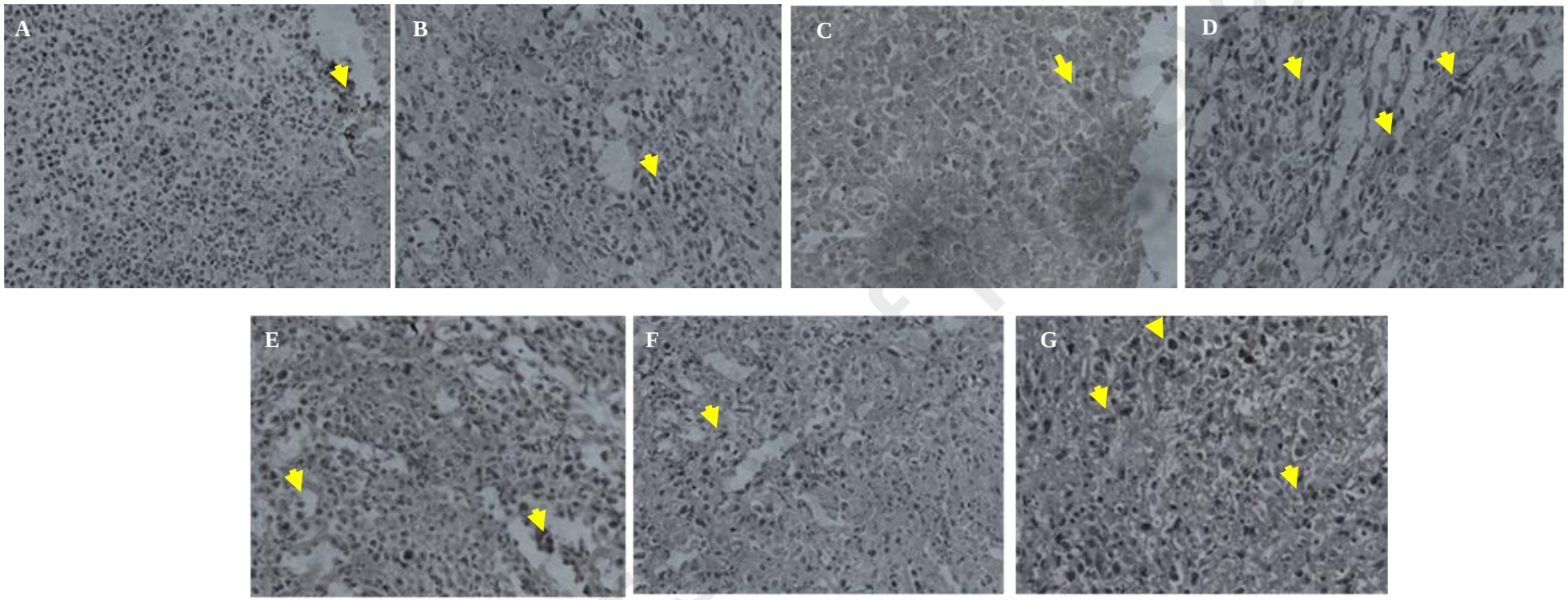


Figure 4.26: Immunohistochemical analysis of the expression of matrix p21 in 4T1 tumour tissue derived from A) placebo; B) MIP treated group; C) ACA treated group; D) CDDP treated group; E) MIP/ACA treated group F) MIP/CDDP treated group; G) MIP/ACA/CDDP treated group. Blue colour indicates nuclei stained with haematoxylin and brown colour indicates specific DAB antibody staining. All images are shown as a representative of three independent replicate at 400x magnification.

4.7.5 Cytokine expression levels

Various cytokines produced by immune cells serve important roles in tumour immunity. Cytokines are low molecular weight proteins involve in mediation of cell communication and regulation of cancer pathogenesis, such as, proliferation, cell survival, differentiation, immune cell activation, cell migration and death. Cytokines can modulate an antitumoural response depending on the tumour microenvironment. At tumour sites, cytokines directly stimulate immune effector cells and stromal cells to enhance tumour cell recognition by cytotoxic effector cells.

A common categorization divides cytokines into pro-inflammatory cytokines which promotes inflammation and anti-inflammatory cytokines which suppress activity of pro-inflammatory cytokines and reduce inflammation (Dinarello, 2000). During chronic inflammation, cytokines can trigger cell transformation and malignancy by controlling their level. Therefore, cytokines has been used as prognosis to detect and monitor the immune system upon treatment. To determine whether MIP, ACA and CDDP treatment induced the release of cytokines that are associated with antitumour immunity, the levels of key cytokines known to have important antitumour activity in the blood serum of the 4T1 tumour-bearing mice in the six different treatment groups were evaluated.

In this study, the Th1 cytokines, namely, IL-2, IL-12, TNF-α and IFN-ɣ as well as the Th2 cytokines, namely, IL-6 and IL-10, were examined. Cytokine assays revealed (Figure 4.27) that the cytokine production profile associated with the exposure of different treatments was distinctly different for each treatment group.

IL-2 is produced by naïve T-cells and helper T cells where it activates cytotoxic lymphocytes (CTL) and natural killer (NK) cells (Hadden, 1988). In this study, all

treatment groups, but not ACA treated mice, inhibited the production of IL-2 when serum from 1st week compared to 5th week treatment. The expression level was increased from 5.71±1.6 pg/ml at the 1st week of treatment to 10.98±1.3 pg/ml at 5th week of treatment. During 5th week, significant increase was achieved in MIP, ACA and triple combination treated mice as compared to placebo groups.

IL-12 is produced by antigen presenting cells (APSs), such as, dendritic cells (DC), monocytes, macrophages and B cells upon Toll-like receptors engagement (Trinchieri *et al*., 1993). Therefore, during infection IL-12 is secreted as an early pro-inflammatory cytokine (Medzhitov, 2001). Upon treatment with MIP, ACA, CDDP and MIP/CDDP, IL-12 level in mice blood serum increased at 5th week compared to the 1st week of treatment. However, its expression reduced in MIP/ACA/CDDP treated mice while the level remained the same in placebo and MIP/ACA treated mice. A significant increase was observed in ACA and CDDP standalone treated groups.

TNF-α is a pleiotropic cytokine with dual important roles, one as a tumour promoter by stimulating cancer cell growth, proliferation, invasion and metastasis. On the other hand, it acts as a tumour suppressor by inducing proliferation, survival, migration and angiogenesis in most cancer cells that are resistant to TNF-induced cytotoxicity (Wang & Lin, 2008). Therefore, TNF-α is also well known as double-edge sword that could be either pro or anti-tumourigenic. In this study, TNF-α expression was comparatively low in all the treatment groups at 1st and 5th week post-treatment except in triple combination drug treated group. In MIP/ACA/CDDP treated group, TNF-α level reached its highest level at around 180±4.2 pg/ml during the 1st week of treatment but reduced to 11.7±1.2 pg/ml at the end of the treatment course. CDDP and MIP/ACA treated mice showed significant reduction in secreted level compared to placebo in the 5th week.

Similar to TNF-α, IFN-ɣ too has a dual role as pro and anti-inflammatory cytokine. Its pro and anti-tumourigenic activities are dependent on the cellular and microenvironment (Zaidi & Merlino, 2011). In this study, upon treatment with MIP/ACA the IFN-ɣ level in blood serum significantly increased at 5th week. However, in the rest of the treatment group IFN-ɣ expression level were reduced at 5th week compared to the levels in the blood serum during the 1st week.

IL-6 is a pleiotropic inflammatory cytokine that plays vital role in cancer progression, including proliferation, migration and angiogenesis (Grivennikov *et al*, 2009; Santer *et al*, 2010). It is produced by a variety of cells including macrophages, monocytes and tumour cells (Nagasaki *et al*., 2014). In the CDDP and MAC treated groups, IL-6 level were reduced while an increased expression was observed in other treatment groups. During the 1st week of CDDP treatment, IL-6 expression reached 80.52±2.5 pg/ml. However, this level reduced to 44.87±1.8 pg/ml at 5th week of treatment. Similarly, around 50 % decrement in expression level was noticed in MIP/ACA/CDDP groups where the IL-6 level was reduced from 104±2.6 to 58±5.5 pg/ml. In, MIP, ACA and MIP/CDDP treated groups IL-6 levels were increased at the end of treatment course where the concentration in blood serum recorded 57±1.0 pg.ml, 63.2±2.3 pg/ml and 71±3.0 pg/ml, respectively. During the 5th week, significant increase in the IL-6 expression was observed in MIP/CDDP treated groups while a significant reduction is seen in MIP/ACA treated mice as compared to placebo at 5th week.

Next, IL-10 is an immune-suppressor and anti-inflammatory cytokine. In all the tested groups, increased IL-10 level was observed compare to placebo. A significant increase of about 85.2 % in the cytokine level was achieved in ACA treated group, followed by CDDP, MIP/ACA, MIP, MIP/CDDP and lastly in MIP/ACA/CDDP treated group. A

significant increase was also noted in ACA, CDDP and triple combination drug treated mice.

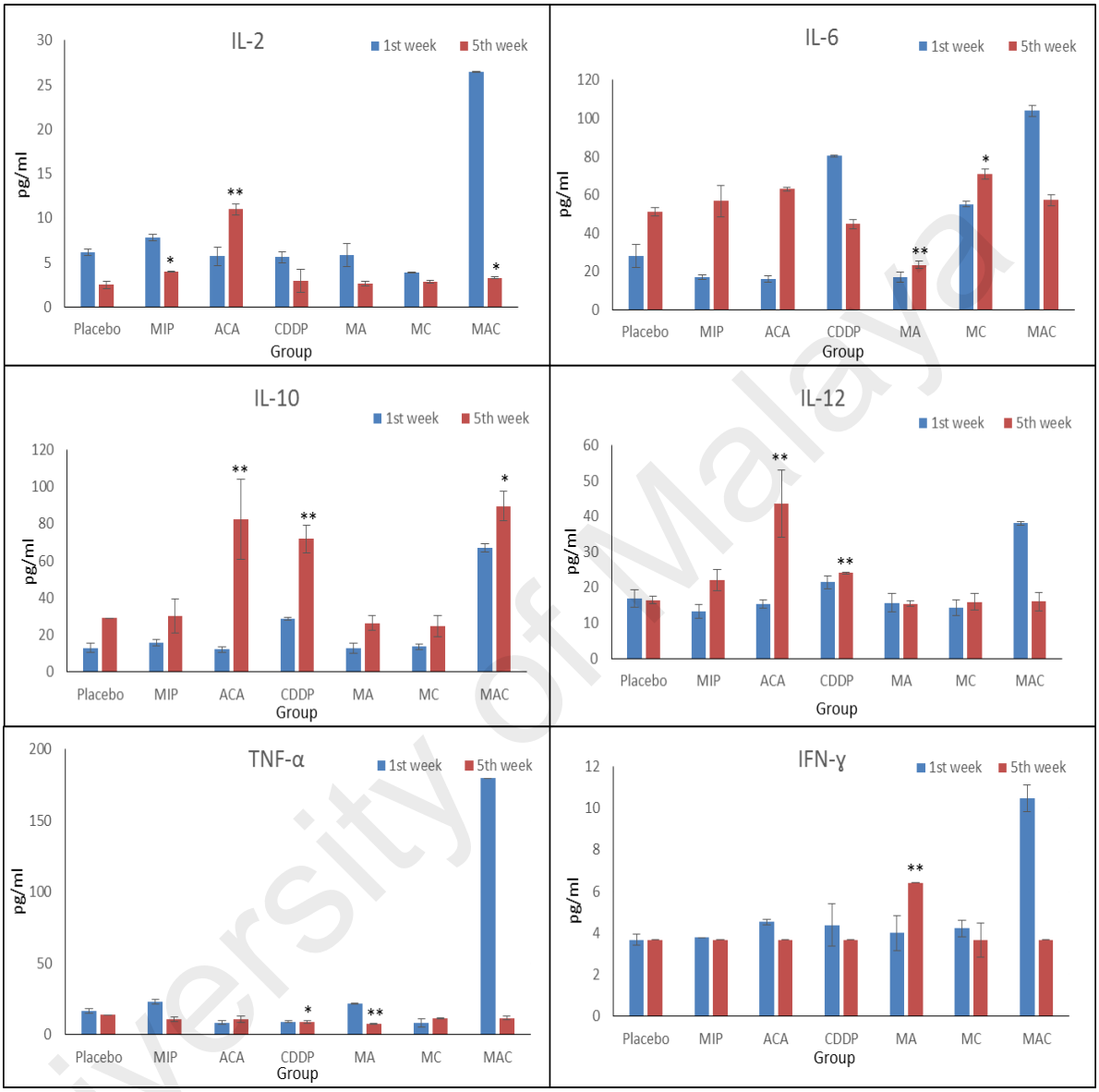


Figure 4.27: Expression levels of cytokines upon treatment using blood serum at 1st and 5th week of the treatment. MA refers to MIP/ACA; MC refers to MIP/CDDP; MAC refers to MIP/ACA/CDDP. Significance between placebo and treatment groups at 5th week are indicated as follows: *p < 0.05; **p < 0.01.

CHAPTER 5: DISCUSSION

Cancer remains to be a great medical challenge worldwide since its diagnosis and prognosis still fails to treat or cure cancer. Even though many studies have been carried out to address the treatment related problems and tackle cancer progression, the attempts often fails due to the complexity of the disease. The cancer cell, originally a normal cell susceptible to genetic alteration, is subjected to multi-step alterations. It behaves as an independent cell, growing without control to form tumour. Tumour grows in a series of steps, starting from hyperplasia (too many cells from uncontrolled cell division), dysplasia (further growth and abnormal changes to cells) and anaplastic (spread over a wider area of tissue, thus they begin to loss their original function) and finally metastasis (when it invades surrounding tissues including bloodstream). Therefore, a single drug to target these numerous genetic alterations usually fails.

Many different treatments have been prescribed to treat different cancers depending on the cancer types, its location, advancement state, patient's health issue and other important parameters. Traditional treatment usually carried out includes surgery to remove solid tumour and radiation to kill remaining cells by damaging DNA to prevent its replication. Next is chemotherapy drug which usually targets cell cycle mechanisms to interfere with cell ability to complete the G1 or S phase. Apart from these approaches, hormonal therapy, immunotherapy, targeted therapy, and stem cell transplant have also been used in the treatment regime. It has widely been accepted that an anticancer drug cocktail instead the single drug/agent improves therapeutic efficacies greatly (Lu *et al*., 2013; Druker, 2003; Siegel *et al*., 2014).

In this present study, it was found that drug combination of natural compound ACA, mycobacterium HKB MIP and commercial FDA anti-cancer drug, CDDP in double and

triple combinations successfully exerts synergistic effect with low dosage /concentration of each drug to induce cytotoxic effect on breast cancer cell, MCF-7. The cell death via inhibition of NF-κB activation in double and triple combinations in both conventional and TNF-α stimulated breast cancer has been highlighted. Apart from *in vitro* analysis, the efficacy of combination treatments was also validated in *in vivo* BALB/c mice model. Mice exposed to combined treatments displayed higher reduction in tumour volume compared to standalone agents. Combined treated mice also demonstrated reduced toxicity in major organs compared to placebo treated mice. The IHC analysis revealed that all three agents in standalones and in combination were not only able to downregulate the major transcription factor, NF-κB activation, but also played a significant role in the expression of NF-κB regulated genes, such as, pro-inflammatory proteins COX-2, HDAC, angiogenic biomarkers VEGF, MMP-9, cell cycle regulators CDK4, p21, apoptotic marker cleaved caspase 3 and histone acetyltransferase p300.

5.1 Agar diffusion assay

Agar diffusion assay is a well-established qualitative analysis which was developed as early as 1940s to test the interaction of drugs with microorganism. This method has been widely used in clinical microbiology laboratories for routine antimicrobial susceptibility testing. It offers many advantages, namely, simplicity, low cost, the ability to test enormous numbers of micro-organisms and antimicrobial agents, and the ease to interpret results (Balouiri *et al*., 2015). Generally, antimicrobial agents would diffuse into the agar and inhibits germination and growth of the microorganism which is shown by zone of inhibition. The diameter of the zone reflects the susceptibility of the bacteria and shows diffusion rate of the drug through the agar medium (Reller *et al*., 2009). In this study CDDP and ACA did not show inhibitory effect against MIP, which suggested that these agents can be used together in combination to obtain a synergistic interaction. In contrast,

a study by Chandrasekar *et al*., 2000 reported the antifungal activity of CDDP and other platinum based drug on *Candida albicans*, a disease-causing pathogen among cancer patients was tested using agar diffusion assay. Only CDDP showed an inhibitory effect as low as 40 mg/ml against *C. albicans* whereas the other platinum-containing drugs did not show any inhibitory effect even at higher concentrations. Therefore, CDDP played two key roles, to inhibit or treat patients with candida infection prior to cancer treatment. The presence of zone of inhibition showed that the compound represses the growth of microorganism. It could therefore, only be used to control or inhibit the growth of microorganism and are not suitable to work together in combination where else an absence of inhibition zone indicate they can work together.

5.2 *Mycobacterium indicus pranii* heat killed bacteria preparation

Bacteria as an anticancer agent is a well-known approach which was initiated by W. Coley and German physicians W. Busch and F. Fehleisen who reported the recovery of neck and other cancers following an infection with *Streptococcus pyogenes* (Nauts, 1980). Apart from *S. pyogenes*, other bacterial species have been found to elicit significant anti-tumour activity *in vitro* and *in vivo,* such as, Lactobacillus species on bladder cancer (Seow *et al*., 2002) and attenuated Salmonella species in murine tumour models (Luo *et al*., 2001). Mycobacteria may be yet another promising species as it has shown a long successful history in treating cancer. For instance, the *bacillus Calmette-Gue'rin* (BCG) vaccine derived from *Mycobacterium bovis* was reported to be effective in treating human bladder cancer (Morales *et al*., 2003).

Bacteria based anti-tumour therapy possess several advantages over chemical based drug. Firstly, some bacteria are able to selectively replicate and accumulate within tumour due to the hypoxia environment and inhibit tumour growth. For instance, *Salmonella*

typhimurium, a facultative anaerobe, has been used as an anti-tumour agent since it can amplify within the tumour mass while inhibiting the tumour growth (Pawelek *et al*., 1997). Next, motile bacteria are able to spread throughout the tumour and help in targeting systemic diseases. They can readily express multiple therapeutic transgenes, such as, cytokines and pro-drug converting enzymes to eradicate tumour mass (Nauts *et al*., 1953).

However, the usage of bacteria in cancer therapy can be a real challenge since its morphology or bacterial content, such as, proteins could be toxic or introduce unnecessary contaminants to human. Therefore, the usage of precise fraction of microbial cell is essential. This is especially so with vaccines as it is of great interest to explore the bacterial factors exposed on the bacterial cell surface to be directly accessed by the immune system. Therefore, in this study instead of using MIP as a whole cell, MIP was fractionated into four fractions: live bacteria (LB), culture supernatant (LS), heat killed bacteria (HKB) and heat killed culture supernatant (HKS). MIP HKB was identified as an active fraction which inhibited two different cancer cells, A549 and CaSki. Therefore, this fraction was selected as an active fraction to induce cytotoxicity effect against various cancer cell lines investigated.

Moreover, the method of HKB preparation also influences the presence of active element in the fraction to induce cytotoxicity effects. While past studies have cited that autoclaving for 20 min at 15 lb/in^2 as the most common heat killing method (Purswani *et al*., 2011; Ahmad *et al*., 2011; Gupta *et al*., 2012), however, this method may denature important and biologically active proteins. This led us to heat MIP at only 60 °C which was found to be sufficient to kill the MIP cultures. When all MIP fractions were cultivated in 7H10 agar, no growth was observed after a week of incubation, with the exception of LB fraction. Thus this confirmed the complete killing of MIP at 60 °C. This method is

recommended because even though MIP cultures were completely heat-killed, other intracellular and extracellular proteins/precursors potentially responsible for its cytotoxicity would likely remain intact at this temperature.

5.3 Cytotoxic effect of standalone drug

5.3.1 MIP

Cytotoxicity level of each fraction was identified using MTT assay where only HKB fraction showed activeness. Among these four fractions, the most widely used fraction being the HKB fraction (Rakshit *et al*., 2011; Gupta *et al*., 2012) followed by the LS fraction (Pandey *et al*., 2011).

Generally, MIP induces patient's immunity by activating $CD4^{+}$T helper cells (Th-1) response to mediate the release of cytokines to promote cell-mediated immunity. Administration of MIP in humans has been reported to be safe and has been practiced in treatment of leprosy (Zaheer *et al*., 1993), tuberculosis (Nyasulu, 2010) and recently in lung cancer (Sur & Dastidar, 2003). Apart from activating the immune system, certain mycobacteria species were also reported to induce a direct cytotoxic effect on cancer cells. For instance, BCG and its cell components could induce cancer cell apoptosis (Morales, 1976) while *M. phlei* or mycobacteria cell wall and DNA are reported to induce anti-tumour activities (Filion, 1999). In accordance with these findings, MIP and its fractions were tested as a potential anti-cancer agent against various human cancer cell lines and proven to show cytotoxicity effect against all the tested cancer cells. According to a previous study on apoptotic cell death in *in vitro* by Pandey *et al*. (2011), 60–70 µl of MIP is required to induce cell death in 40–45 % mouse peritoneal macrophages while in this study, 60–70 µl/(1.0×10^{6} MIP cells/ml) of MIP HKB induced 75 % cell death. This

clearly showed a significant reduction in MIP dose when a 60 °C heat kill technique was applied compared to the usual autoclave heat kill technique.

This study also identified that cancer cell death was induced via apoptosis in MCF-7 and ORL-115 cells as confirmed through PARP and DNA fragmentation assays. Apoptosis is a cell suicide mechanism to remove redundant, damaged, or infected cells through a group of caspases activation. These caspases are grouped into initiator (caspases-2, -8, -9 and -10) and effector (caspases-3, -6 and -7) caspases. Effector caspases are responsible for dismantling of necessary cell components, which results in morphological and biochemical changes that characterize apoptotic cell death as cytoskeletal rearrangement, cell membrane blebbing, nuclear condensation and DNA fragmentation. The DNA fragmentation observed in MCF-7 cells, a caspase-3 deficient cell line, was most probably due to other effector caspases, such as, caspase-7 activation (Margaret *et al*., 2002).

5.3.2 ACA

ACA, a natural phenyl-propanoid induces both anti-proliferative and anti-apoptotic effects on tested cancer cell lines with no cytotoxic effects on HMEC normal human breast cells (Awang e*t al*., 2010). It induced cytotoxicity in a dose and time dependent manner which is a convenient factor as this allows the manipulation and employment of lower drug doses with longer exposure time to reduce toxicity and side effects on non-cancerous tissues. On the contrary, higher drug doses with shorter exposure periods would also be beneficial on assays involving protein and gene expression studies. This is because, these assays often investigate drug mechanism and are generally conducted shortly upon drug exposure to capture intracellular molecular changes and events. Previous studies have shown, ACA induced cytotoxicity effect and caused apoptotic cell death via dysregulation of NF-κB (In *et al*., 2012; Arshad *et al*., 2015).

5.3.3 CDDP

CDDP is a potent chemotherapeutic agent, displays clinical activity against various solid tumour and kills cancer cells through the formation of covalent bifunctional adducts. It has been clinically used against a variety of cancer types including ovaries, testes, solid tumour of head and neck. This FDA approved platinum drug is the key drug for small cell lung cancer, SCLC (Abrams *et al*., 2003). However, in this study, the least sensitivity against CDDP was observed in non-small cell lung cancer (NSCLC) cell lines, A549 and SK-LU-1. In other cancer cell lines, higher IC_{50} range were obtained compared to ACA and MIP standalone treatments. This is probably due to its nature as CDDP might work better as adjuvant therapy rather than standalone drug to inhibit cancer cell growth.

Therefore, combination of CDDP with other agent/ drug is necessary to obtain desired effect. For example, CDDP paired with paclitaxel against gastric and esophagogastric junction adenocarcinoma (Kim *et al*., 1999), with Tegafur-uracil (UFT) in non-small cell lung carcinoma (Ichinose *et al*., 2000), with doxorubicin against advanced carcinoma of salivary gland (Dreyfuss *et al*., 1987) and with gemcitabine for advanced biliary cancer (Valle *et al*., 2010). CDDP has also been combined with vitamin D to work against squamous cell carcinoma (Light *et al*., 1997) and colon cancer (Milczarek *et al*., 2013). Another plus point is that, it is often used in combination due to its toxicity effect when given as single drug treatment. CDDP at higher concentration can be toxic as it causes hepatotoxicity (Santos *et al*., 2007), cardiotoxicity (Al-Majed *et al*., 2006), nephrotoxicity (Arany & Safirstein, 2003), ototoxicity, gastrotoxicity, myelosuppression, allergic reactions (Hartmann & Lipp, 2003) and reduction in the body weight. Therefore, reduced concentration would be seen as a safe decision and can be achieved via combination treatment.

In this study, the cytotoxicity effects of all three drug/agents were less potent towards non-cancerous cells based on their high IC_{50} values. The difference in MIP's selectivity between cancerous and non-cancerous cells may be due to the difference in growth rate of cells upon distinct cell surface receptors (Escribano *et al*., 2000). Moreover, rate of killing between cancer cells varies, hence attaining various IC_{50} values. This is probably due to the way these cancer cell lines turns cancerous. For example, the expression level of certain genes involved in drug efflux and influx within the cell would govern the exposure time for each cancer cell line to react towards tested drug/agent. The intracellular balance between tumour suppressor genes pushing towards apoptosis against various oncogenes working towards anti-apoptosis and proliferation was another likely reason to create a diversified environment capable of influencing the different outcome of each cancer cell type reaction towards an anti-cancer drug. The activation extent of established expression systems involved in chemo-sensitivity such as the NF-κB pathway was also a possible cause explaining how resistant a cancer type is towards a particular drug. For example, it was previously reported that NF-κB regulated glutathione S-transferase gene, involved in metal metabolism, could potentially reduce the efficacy of CDDP by reducing squamous cell carcinoma chemo-sensitivity (Nishimura *et al*., 1996). Lastly, the aggressiveness of each cancer genotype would also without doubt, govern the minimal dosage of drug (MIP, ACA and CDDP) required to achieve the desired IC_{50} levels.

5.4 Synergistic effects of MIP, ACA and CDDP

MIP, ACA and CDDP as standalone drug was proven to induce cytotoxicity effect against all the cancer types tested earlier. However, their combinatorial effect with other compounds/drugs cannot be ruled out. Previous study has reported ACA enhanced cytotoxic effects of CDDP in a synergistic interaction in human oral tumour xenograft

with minimal body weight loss (In *et al*., 2012). CDDP is frequently used in combination with one, two, three, or even four other drugs, with positive results. The intention is for the drugs to work together, producing synergistic or at least additive effects in killing the cancer cells, while producing no additional side effects. On the other hand, the efficacy of MIP in combination with other drugs, have not been reported. Since MIP, ACA and CDDP are drug/agents that individually display anti-tumour activity towards cancer, double and triple combinations of these drugs/agents may translate into improved therapies. Moreover, immunotherapy approach that reactivates the weakened host immune response against the malignancy could be a promising anticancer treatment when used alone or in combination with other anticancer chemotherapeutics.

Thus, in this study ACA in combination with CDDP and MIP was tested to treat cancer more efficiently. Its combinatorial effect in double and triple combination is tested in order to identify the synergistic interaction when lower dosage of each compound is applied.

Combination chemotherapy provides the opportunity to minimize metabolic and clinical side effects due to usage of low doses in comparison to single agent therapy (Sica, 1994). While the cancer adaptation process can be delayed when multiple drugs with different molecular targets are applied, multiple drugs which targets one single cellular pathway would be able to function synergistically for both higher therapeutic efficacy and target selectivity (Lee & Nan, 2012). Development of drug resistance in tumour cells can also be overcome by using combination drug therapy (Szakacs, 2006). Moreover, the advantages of combinations include the ability to replace current expensive anti-cancer therapies through the use of cheaper drug cocktails (Kashif *et al*., 2015).

In this study, triple combination with reduced dosage at IC_{10} exerted synergism compared to higher dosage at $IC_{25,}$ which showed the type of interaction is dependent on the concentration and ratio of combined drugs. Similar result was observed in a study by Pavillard *et al*. (2001), when camptothecin and doxorubicin were exposed simultaneously to glioma cells at a molar ratio of 5:1, strong antagonism was observed, whereas a 1.5:1 ratio resulted in synergistic activity. These results demonstrate that better effects could be acquired in combination therapies with lower doses of each drug compared with higher doses. Therefore, individual toxicities associated with higher doses could be reduced.

Reports have shown that multi-targeted therapies have a higher success rate in inducing cytotoxicity and tumour clearance compared to mono-targeted therapies. Natural products such as curcumin was shown to enhance effects of chemotherapeutic drug, gemcitabine by sensitizing human bladder cancer cell and induces apoptotic effects through NF-κB inactivation (Kamat *et al*., 2007). Similarly, 1'S-1'-acetoxyeugenol acetate (AEA) a phenylpropanoid in combination with paclitaxel chemosensitizes human breast cancer cells and enhances its apoptotic effects (In *et al*., 2011). Similarly, in this study, MIP/ACA/CDDP in combination was able to enhance cytotoxicity effects against cancer cells. Thus, it can serve as a promising therapeutic regime for further *in vivo* development in orthografted animal models to further validate its anti-cancer and immune-potentiating systemic effects.

5.5 Drug combination in relation to the NF-κB pathway

The constitutive activation of NF-κB is associated with the growth and survival of cancer cells (Guttridge *et al*., 1999). Several studies have shown that, chemo-resistance is often contributed by the activation of NF-κB by chemotherapeutic agents (Nakanishi & Toi, 2005). Thus a strategic approach to tackle cancer development is to formulate anticancer

drug which targets NF-κB suppression. It was shown that combining a NF-κB inhibitor with an anticancer drug could enhance overall anti-tumour responses (Chawla *et al*., 2003; Bauer *et al*., 2007). For example, inhibition of NF-κB increased the efficacy of a variety of chemotherapeutic agents including paclitaxel (Mabuchi *et al*., 2004), etoposide, doxorubicin (Arlt *et al*., 2001), cisplatin (Mabuchi *et al*., 2004b), 5-FU (Uetsuka *et al*., 2003), irinotecan, CPT-11, and camptothecin (Sharma *et al*., 2007), thereby potentiating apoptosis.

In the past, it has been reported that ACA inhibited cellular invasion through the suppression of NF-κB regulated gene products (Ichikawa *et al*., 2005). In 2005, Ito *et al* reported ACA from *Languas galangal* significantly inhibited the serine phosphorylation and degradation of IκBα in a time dependent manner in myeloid leukemia cells leading to the prevention of NF-κB nuclear translocation and its accumulation within the cytosol. On the other hand, microarray global gene expression data on ACA extracted from *Alpinia conchigera* treated cancer cell revealed that NF-κB inactivation played a key role which led to a series of apoptosis inducing events and suppress NF-κB activity through IKKα/β suppression (In *et al*., 2012).

On the other hand, heat killed MIP was shown to inhibit NF-κB activation in melanoma cancer therapy (Halder *et al*., 2015). Even though a number of studies have reported MIP as an immune-stimulator with the ability to inhibit proliferation of cancer cells, the mechanism or mode of action was not reported (Sur & Dastidar, 2003; Chaudhuri & Mukhopadhyay, 2003). Since, NF-κB plays an important role in various biological processes including apoptosis, stress response, immunity, and inflammation, the immune-potentiating effect of MIP together with NF-κB could enhance anti-tumour activity through the immune system.

In this study, we have shown that MIP in combination with ACA or/and CDDP was capable of inhibiting NF-κB activation through blocking p65 cytoplasm translocation to the nucleus. A number of studies have shown dysregulation of NF-κB inactivation in combination with chemotherapy. For example, natural products such as curcumin is shown to enhance effects of chemotherapeutic drug, gemcitabine by sensitizing human bladder cancer cell line and induces apoptotic effects through NF-κB inactivation (Kamat *et al*., 2007). Similarly, 1'S-1'-acetoxyeugenol acetate (AEA) a phenylpropanoid in combination with paclitaxel chemosensitizes human breast cancer cells and enhances its apoptotic effects (In *et al*., 2011).

Similarly, in this study, MIP/ACA/CDDP in combination was able to enhance cytotoxicity effects against cancer cells. Thus, it can be served as a promising candidate for further *in vivo* development in orthografted animal models to further validate its anti-cancer and immune-potentiating systemic effects. Apart from inhibiting cells basal NF-κB activation, MIP, ACA, and CDDP in combination was also shown to suppress TNF-α induced NF-κB activation.

5.6 *In vivo* animal study

Upon confirming the consistency of standalone and combination drug interaction in *in vitro* chemo-potentiating and apoptosis-inducing effects, *in vivo* studies were conducted using BALB/c mice model. These inbred mice have been used for more than 50 years to investigate tumour growth and treatment responses (Campbell *et al*., 2014). They are the preferred experimental model and demonstrate Th-1 biased immune responses. BALB/c mice are also very sensitive to carcinogens, and can develop lung tumours, reticular neoplasms, renal tumours, and others. Since, these mice have a fully competent immune response, they were selected as a most suitable animal model in this study to identify

immune-potentiating effect of MIP as a standalone or in combination treatments. Many studies involving investigation of immunological ability of MIP have been carried out using this mice model (Faujdar *et al*., 2011; Rakshit *et al*., 2011; Adhikari *et al*., 2012). But however, it requires mouse-strain specific murine tumour models. Therefore, 4T1 mouse breast cancer cell line was selected instead of MCF-7. If a mismatched cell line was introduced, it will prevent or cause a major source of variation in tumour formation and usually leads to easy rejection.

However, the 4T1 cell line is murine stage IV breast cancer that very closely mimics human breast cancer (ATCC), which is an added advantage in this study. Subcutaneous injection of 4T1 in mammary pad of BALB/c was successful when tumour development was noticed from day 7.

5.7 Post *in vivo* analysis

In the *in vivo* study, combination treatment of MIP/ACA, MIP/CDDP and MIP/ACA/CDDP showed decreased tumour growth at 5^{th} week as compared to placebo groups. While minor weight loss was observed in MAC treated group, all the other groups maintained their weight in a range of 15-20 g. Tumour volume is calculated to identify the tumour burden and to evaluate therapeutic responses. The combined regime reduced tumour size more efficiently while maintaining body weight throughout treatment period. Next, toxicity in major organ was observed in all treatment groups including placebo which suggested that the toxicity is not caused by the different treatment regime but due to the aggressive 4T1 tumour. 4T1-induced metastasize spontaneously from the primary site in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone (Pulaski & Rosenberg, 1998; Lelekakis *et al*., 1999). Interestingly, in combination treated mice, reduced level of toxicity was observed, promising the

treatment with MIP, ACA and CDDP not only reduced tumour volume but also played key role as anti-metastatic agents.

5.7.1 NF-κB activity and its inflammatory expression level upon treatment in *in vivo* animal model

NF-κB signalling pathway has become a potential target for pharmacological interventions since it regulates the expression of over 500 genes which are involved in cellular transformation, survival, proliferation, invasion, angiogenesis, metastasis, and inflammation. A number of studies has shown NF-κB plays a role as a link between inflammation and cancer progression (Haefner, 2002; Aggarwal, 2004; Karin & Greten, 2005), making NF-κB essential to and a potential drug target in hematological malignancies and solid tumours (Shaffer *et al*., 2002; Panwalkar *et al*., 2004). Next, IHC analysis was carried out in this study to validate NF-κB activity using its subunit p65 and its regulated inflammatory biomarkers, such as, COX-2, HDAC2, p300, cleaved caspase-3, p21, CD1, CDK4, VEGF and MMP-9 in tumour biopsies. IHC is a technique used to determine antigen distribution in tissue using monoclonal or polyclonal antibodies in tumour and infection studies.

Constitutively active NF-κB subunit p65 was inhibited in all the groups while the level was maintained in MIP/ACA group. A variety of agents, such as, phorbol esters, tumour necrosis factor (TNF) and hydrogen peroxide (Grili *et al*., 1993) can induce NF-κB activation. Its activation requires changes as phosphorylation, poly-ubiquitination, and subsequent degradation of its inhibitory subunit, IκBα. IκBα phosphorylation is carried out by IKKα/β/ɣ. Inhibiting IκBα phosphorylation can lead to inhibition of NF-κB transcriptional activity (Liu *et al*., 2000). Hence, one strategy for inhibiting NF-κB

activity is through the blocking of IKK activation. Therefore, the phosphorylated level of IKKα/β was also analyzed in IHC which revealed similar pattern as the p65 expression. In this study, for the first time MIP, ACA and CDDP as standalone and in MIP/ACA combination are shown to block IKK phosphorylation and subsequent p65 inactivation in treated groups compared to placebo. Moreover, agents that inhibited NF-κB have been shown to reduce growth (Brown *et al*., 2008) which is reflected in tumour reduction of treatment groups. This was in agreement with earlier study by Li *et al*., 2013, where tumour growth suppression observed with inhibition of both NF-κB p65 and IKK expression upon treatment with garcinol (camboginol) extracted from dried rind of the fruit *Garcinia indica*.

Treatment with combination agents/drugs is seen to mediate anti-tumour activity in *in vivo* by modulating the expression of numerous inflammatory biomarkers protein, such as, down-regulation of COX-2, VEGF and MMP-9 expressions against tumour cell proliferation, invasion and angiogenesis. Invasion and angiogenesis are two critical events for tumour metastasis regulated by NF-κB (Bharti & Agarwal, 2002). Overexpression of COX-2 has been reported to stimulate cancer proliferation, inhibit apoptosis and induce angiogenesis (Romano & Claria, 2003). Similarly, in a study by Gupta *et al* (2010), reduced expression of COX-2 and MMP-9, accompanied by reduced NF-κB activation was observed in mammary tumours isolated from rats when treated with resveratrol, a phytoalexin present in grapes.

One of the major characteristic of malignancy is the dysregulation of proliferation. Unlike normal cells, cancer cells lack regulation to balance growth and antigrowth signals. Therefore, they become insensitive to antigrowth signals. Their cell growth is controlled by cell cycle regulators at G1/S phase especially CDKs. Combination and standalone

treatment successfully regulated cell cycle by the upregulation of histone acetylase p300 which act as a double edged sword for tumour growth depending on the cell types and signaling pathways. It was reported that the p300/CBP-pCAF protein complex can arrest cell cycle progression (Yang *et al*., 1996) and might regulate target genes that are involved in controlling the G1/S transition, such as $p21^{WAF1}$ (Missero *et al*., 1996). In addition, CD1 with its binding partner CDK4 forms active complex to promote cell cycle progression. Their expression in this study is in moderate level. However, HDAC2 expression is downregulated only in MIP and ACA treated groups, while the level is unexpectedly increased in the rest of treatment groups. The expression of all four markers as cell cycle regulators may depend on the pathophysiological milieu of the cell type.

Apoptosis regulation by cleaved caspase-3 was observed in treatment groups. Increased apoptosis in double and triple combination groups showed enhanced apoptotic effect compared to placebo and single drug treated groups. This is due to the activation of intrinsic apoptosis by MIP and extrinsic apoptosis by ACA and CDDP. Therefore, treatment with the combination drugs/agents would combine effect of these two death pathways to enhance the apoptotic effects in breast cancer cells.

5.7.2 Cytokine expression level upon treatment in *in vivo* animal model

Cytokines involve in many aspects of cancer, including development, advancement, treatment, and prognosis as well as to monitor effectiveness of cancer treatments. Recent evidence indicates that cytotoxic anticancer agents also affect the immune system, contributing to tumour regression (Correale *et al*., 2006; Prete *et al*., 2008). Effect on immune system upon treatment was investigated via cytokine expression level on the 5^{th} week.

IL-2 is an immune modulator, a protein made by T-helper cells when white blood cells are stimulated by an antigen. It has been approved by FDA for clinical application of certain cancer (Liu *et al*., 2006). IL-2 is a main cytokine that is involved in proliferative stimulation of activated T-cells, thus improves the body's natural response to disease. Moreover, stimulation of IL-2 can promote induction, activation and reproduction of all kind of effector cells. Observing IL-2 serum level is also helpful in monitoring therapeutic effect (Eugster *et al*., 1996; Driver, 2004). An increase of IL-2 level was observed in all treatment groups at the 5th week, thus regress tumour cells while enhancing the immune system. A study by Rodella *et al*., (1997) suggested IL-2 contributed to the reduction of tumour cells through the generation of lymphokine-activated killer cells (LAK).

Similarly, TNF-α showed decreased expression in all the treated groups while a significant decrease was achieved in CDDP and MIP/ACA treated mice. TNF-α is a pleiotropic cytokine with wide range of biological effects. It is secreted by inflammatory cells, which are involved in inflammation-associated carcinogenesis. Mounting evidence indicated that TNF-α promoted genesis and growth. For instance, Popivanova *et al*. (2008), has shown in colon carcinogenesis mice model lacking TNF-α function, reduced colonic inflammation and tumour formation. In contrast, the increased TNF-α expression levels in pre-cancerous cells was associated with the progression of breast cancer (García-Tuñón *et al*., 2006).

IL-6 secretion is often involved in severe cancer progression. Elevated IL-6 in the serum of prostate cancer patient induced tumour progression and metastasis (Adler *et al*., 1999; Drachenberg *et al*., 1999) while in breast cancer, IL-6 is involved in oncogenic transformation, invasion, and metastasis (Sansone *et al*., 2007). They also eventually cause enhanced ability to invade the extracellular matrix and increased drug

resistance (Sehgal & Tamm, 1991). A significant reduction in IL-6 expression upon MIP/ACA combination in this study revealed the ability to suppress tumour progression and the possibility to inhibit aggressive tumour progression and metastasis.

IL-10 is an immune-suppressor which can inhibit NF-κB activation and consequently inhibits the production of pro-inflammatory cytokines including TNF-α, IL-6, and IL-12. Therefore, it is not a surprise that IL-10 can inhibit tumour development and progression (Schottelius *et al.*, 1999; Hoentjen *et al*., 2005). This complex activity of IL-10 has also been proven in this study, with a decreased level of IL-6 and TNF-α.

Interleukin-12 (IL-12) is a pleiotropic cytokine which interconnects the innate and adaptive immune responses by inducing IFN-γ production primarily from natural killer and T cells. IL-12 expression is elevated in all the treatment groups which showed the anti-tumour effect in 4T1 advance breast cancer. However, the expression in MIP/ACA/CDDP treated group is reduced between the 1st and 5th week. Suppression of IL-12 production is mediated by the cytokines IL-10, and TGF-β, as well as prostaglandin E_2 (PGE_2) that is produced by various cancers (Mitsuhashi *et al*., 2004). Within a tumour environment, IL-12 suppression occurs due to T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) (Alderton, 2012). Moreover, immunotherapy using IL-12 administration in 4T1 tumour-bearing mice has resulted in the reduction of tumour size and reduced metastasis in the lung while displaying significant prolonged survival time (Rakhmilevich *et al*., 2000). IFN-ɣ along with lymphocytes has been shown to protect against development of sarcomas and epithelial carcinomas (Kaplan *et al*., 1998). It enhanced cell immunogenicity and elimination of tumour by lymphocytes via up-regulation of MHC class 1 antigen-processing and presentation pathway (Dunn *et al*., 2005).

Overall, an increase of anti-inflammatory cytokine expression (IL-10, IL-2, IFN-ɣ) while decrease in TNF-α, IL-12 and IL-6 levels has been seen in the treatment groups which suggested successful therapy in 4T1 aggressive breast cancer treatment using the combination regime. Although, in certain groups the levels were opposite it may be due to the cancer type used in this study and the complex microenvironment, serving as a model imitating the appropriate physiological, immunological and biomechanical components of heterogeneous growth seen in cancer.

CHAPTER 6: CONCLUSION

The main purpose of this study was to determine combination therapy of natural compound, ACA, mycobacterium, MIP and commercial drug, CDDP in double and triple combinations in order to chemo- and immuno-potentiate to eradicate targeted cancer specifically breast cancer in synergistic drug interaction. *In vitro* pre-screening biological activity using MTT assay was carried out in various cancer cell lines as standalone and as well in combination of MIP/ACA, MIP/CDDP and MIP/ACA/CDDP. Cancer cell line with synergistic drug interaction, MCF-7 was selected as a promising model for validation using *in vivo* animal model. Furthermore, in this study MIP was fractionated into MIP HKB, LB, LS and HKB, then the active fraction was identified using MTT cytotoxicity assay. Among the four fractions, only MIP HKB showed cytotoxicity effect and viable cell reduction randomly in CaSki and A549 cancer cell lines. Subsequently, the cytotoxicity effect of MIP HKB as a standalone regime was further analyzed in various cancer cell lines using DNA fragmentation and PARP assays. It confirmed occurrence of apoptotic cell death in the tested breast cancer cell line (MCF-7) and oral cancer cell line (ORL-115). Since previous study has shown ability of ACA to induce apoptotic cell death via NF-κB inactivation, this study also focused on suppression of this pathway upon treatment with combination drugs. Western blot analysis in double and triple combination treated breast cancer cells showed activation of intrinsic apoptosis as the apoptotic proteins, Apaf-1 and caspase-9 were expressed upon treatment. Besides that, NF-κB inactivation was monitored using p65, IκBα and p-IκBα. Following preliminary cytotoxicity assay and western blot analysis, this study is the first to show that bacteria (MIP) in combination with natural extract ACA and/or CDDP suppressed the activation of NF-κB. In addition, this study also successfully demonstrated the combination able to potentiate anticancer effect in *in vivo* animal model. *In vivo* animal model using BALB/c mice showed tumour regression and maintained regular body weight throughout the

treatment. Most importantly, immunohistochemistry results provided conclusive evidence indicating that combination regimens were able to downregulate NF-κB activation and also reduced the expression of NF-κB regulated pro-inflammatory proteins. Treatment with combination agents/drugs is seen to mediate anti-tumour activity in *in vivo* by modulating the expression of numerous inflammatory biomarkers protein, such as, downregulation of gene expression in tumour cell proliferation, invasion and angiogenesis such as COX-2, VEGF and MMP-9. Moreover, cytokine expression study revealed role of immune system upon combination treatment. An increase of anti-inflammatory cytokine expression (IL-10, IL-2, IFN-ɣ) while decrease in TNF-α, IL-12 and IL-6 levels has been seen in the treatment groups which suggested successful therapy in 4T1 aggressive breast cancer treatment using combination regimens. Therefore, this study indicated that combined drug regimens were successful in enhancing the effect by preventing the dose-limiting toxicity in breast cancer treatment. It was therefore concluded that, MIP, ACA and CDDP in combination serves as a promising candidate for further development and subsequent clinical trials involving patients with breast cancer. Future studies also should further investigate role of drug combination proposed in this study in the occurrence of metastases, since cancer death is often correlated to metastases. In addition, synergistic effect of these three drugs/agents should be further validated in other human cancer types such as cervical, lung, oral and prostate.

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LIST OF PUBLICATIONS

1. **Subramaniam, M**., In, L.L., Kumar, A., Ahmed, N., & Nagoor, N.H. (2016). Cytotoxic and apoptotic effects of heat killed *Mycobacterium indicus pranii* (MIP) on various human cancer cell lines. *Scientific Reports*, *6*, 19833. (Published) Impact factor 5.2.

2. **Subramaniam, M**., Su Ki, L., In, L.L., Awang, K., Ahmed, N., & Nagoor, N.H. Inactivation of Nuclear Factor κB by MIP-based drug combinations augments cell death of breast cancer cells. Drug design, development and therapy. Accepted (2017). Impact factor 3.2.