STUDY OF *PITHECELLOBIUM JIRINGA* (JACK) PRAIN ON CARCINOGENESIS AND ANGIOGENESIS MECHANISTIC PATHWAY

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By

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This thesis is dedicated to....

My parents

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-sulphonic acid)
ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
CCD-18Co cells	Normal colonic cells
CD20	B-lymphocyte antigen
CET	Cetrimide
cfu/g	Colony forming units per gram
CMG-2	Capillary morphogenesis gene-2
CO_2	Carbon dioxide
DEVD-,	Amino acid sequence substrate for caspase 3/7
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EBV	Epstein-Barr Virus
ECGS	Endothelial cell growth supplement
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immnuosorbent assay
EMT	Epithelial-mesenchymal transition

EtOH extract	Ethanolic extract
EW extract	50% ethanolic extract
FBS	Foetal bovine serum
Fc	Fragment crystallisable region of an antibody
FDA	Food and Drug Administration
FGF	Fibroblast growth factor -3
FTIR	Fourier Transform Infra-Red
g	Gram
GC-MS-TOF	Gas chromatography-mass spectrometry-time of flight
H_2SO_4	Sulfuric acid
h	Hour
HCl	Hydrochloric acid
HCT 116 cells	Colorectal cancer cells
HepG2 cells	Hepatoma carcinoma
HeLa cells	Human cervical carcinoma
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor 1
HPTLC	High Performance Thin Layer Chromatography system
HPV	Human papillomavirus
HREs	Hypoxia-response elements
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Inhibitory concentration of 50%

IFN	Interferon
IL	Interleukin
-LEHD	Amino acid sequence substrate for caspase 8
LETD-	Amino acid sequence substrate for caspase 9
M199	Earles salt medium
MCF 7	Breast cancer cells
MCP-1	Monocyte chemotactic protein-1
MEM	Minimum Essential Medium
MeOH extract	Methanolic extract
mg	Milligram
mg/ml	milligram per millilitre
min	Minute
ml	millilitre
mM	Millimolar
MMPs	Matrix metalloproteinase
mRNA	messenger Ribonucleic acid
MSA	Mannitol salt agar
MTD	Maximum tolerated dose
MTT	3-(4,5-Dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
MW extract	50% methanolic extract
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NIST	National Institute of Standards and Technology
nm	Nanometer
PBS	Phosphate buffered saline
PDGF-BB	Platelet-derived growth factor-B
PEG 4000’	Polyethylene glycol 4000
pg	picogram
PIGF	Placental growth factor
P. jiringa	*Pithecellobium jiringa*
PS	Penicillin-streptomycin solution
Psi	Pound-force per square inch
ROS	Reactive oxygen species
STMs	Signal transduction modulators
TEMs	Tumour endothelial markers
TGF-β	Transforming growth factor-β
TLC	Thin layer chromatography
TNF	Tumour-necrosis factor
Trolox	6-hydroxyl 2,5,7,8-tetramethylchroman-2-carboxylic acid
V	Volt
VEGF	Vascular endothelial growth factor
Wa extract	Aqueous extract
XLD	Xylose lysine deoxycholate agar
5-FU	5-fluorouracil
µg/ml	microgram per millilitre
µm	Micrometer

APPENDICES

ABSTRAK

Kajian *Pithecellobium jiringa* (Jack) Prain Ke Atas Laluan Mekanistik Karsinogenesis dan Angiogenesis

Dalam kajian ini, potensi kulit buah *P. jiringa* sebagai anti-tumor telah diselidik. Satu siri penyelidikan untuk menilai kesan anti-tumor dan komponen-komponen yang memainkan peranan dan sasaran biologikal yang menyumbang kepada kesan anti-tumor telah dijalankan. Dalam bahagian pertama kajian ini, satu siri pengekstrakan kulit buah *P. jiringa* dari pelarut 100% etanol (EtOH), 50% etanol (EW), 50% metanol (MW), 100% metanol (MeOH) dan 100% air (Wa) telah dikembangkan melalui teknik maserasi. Ekstrak-ekstrak tersebut telah memberi kesan sitostatik yang kuat kepada sel-sel endothelium pembuluh umbilikal (HUVEC) (IC_{50} 0.60 ± 0.15 µg/ml dan 3.01± 0.05 µg/ml untuk EtOH and EW) dan aktiviti sitotoksik terpilih yang kuat ke atas sel-sel kanser kolorektal (HCT 116) (IC_{50} 5.54 ± 0.13 µg/ml dan 12.63 ± 0.17 µg/ml untuk EtOH and EW). Kajian anti-kanser *in vitro* ke atas sel-sel HCT 116 telah menyebabkan kematian sel melalui laluan ekstrinsik. Ekstrak-ekstrak EW dan EtOH didapati menyebabkan gangguan pada penghijrahan dan pembentukan tiub endotelium di dalam sel-sel HUVEC. Ekstrak-ekstrak ini juga telah menyebabkan perencatan *angiogenesis* di dalam ujian tisu terasing dan merencat pengungkapan *VEGF* yang merupakan penanda *angiogenesis* utama. Aktiviti *anti-angiogenic* juga telah dicerap secara *in vivo* di dalam embrio telur ayam yang subur. Kedua-dua ekstrak EW dan EtOH menunjukkan kelimpahan unsur-unsur fenolik yang memberi sifat antioksidan yang kuat. Sifat yang kedua mungkin faktor utama menyumbang kepada aktiviti *anti-angiogenic* yang dicerap. Hasil keputusan

daripada kajian-kajian ini mencadangkan bahawa ekstrak EW dan EtOH mungkin berguna untuk terapi kanser terutama sekali terhadap tumor yang bergantung tinggi kepada *angiogenesis* seperti kanser kolorektal.

STUDY OF *PITHECELLOBIUM JIRINGA* (JACK) PRAIN ON CARCINOGENESIS AND ANGIOGENESIS MECHANISTIC PATHWAY

ABSTRACT

In this work, the anti-tumour potential of *P. jiringa* fruit rind was investigated. An array of studies was carried out to evaluate its anti-tumour effect and characterised the components at play and the biological target(s) that contribute to the anti-tumour effect. In the first part of this work, a series of solvent extract(s) of the *P. jiringa* fruit rind namely 100% ethanol (EtOH), 50% ethanol (EW), 50% methanol (MW), 100% methanol (MeOH) and 100% water (Wa) was developed via maceration technique. The extracts gave strong cytostatic response on human umbilical vein endothelial cells (HUVEC) (IC_{50} 0.60 ± 0.15 µg/ml and 3.01± 0.05 µg/ml for EtOH and EW respectively) and strong selective cytotoxic activity on colorectal cancer cells (HCT 116) (IC_{50} 5.54 ± 0.13 µg/ml and 12.63 ± 0.17 µg/ml for EtOH and EW respectively) The *in vitro* anti-cancer study on HCT 116 cells showed that both EtOH and EW extracts caused cell death via extrinsic pathway. In the HUVEC cells we found that both EW and EtOH disrupted cell migration and endothelial tube formation. The extracts caused marked inhibition of angiogenesis in isolated tissue assay and inhibited the expression of VEGF which is the key marker of angiogenesis. The anti-angiogenic activity was also observed *in vivo* in fertilised chicken egg embryo. Both EW and EtOH showed abundance of phenolic compounds giving rise to strong antioxidant property. The latter may be the key factor that

contributes to the anti-angiogenic activity observed. The result of this study suggests that EW and EtOH extracts may be useful in cancer therapy particularly towards the highly angiogenic dependent tumours such as colorectal cancer.

CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer refers to malignant diseases affecting various parts of the body. This disease is characterised by rapid, uncontrolled abnormal cell formation leading to tumour formation which may further proliferate throughout the body (Vanhoecke et al. 2005). Cancer has become a major public health concern in most regions of the world. Cancer statistics comprised cases reported on both men and women in developed and developing regions shows that incidence rates of cancers in developed regions are higher than in developing regions; however, the mortality rates are typically higher in developing regions (Parkin et al. 2005).

In 2002, the number of new cancer cases reported was 10.9 million, with 6.7 million deaths and estimation of 24.6 million persons living with cancer worldwide (Parkin et al. 2005). Among all cancers, the major five according to the highest incidence rates recorded were lung cancer with 1.35 million cases, breast cancer of 1.15 million cases, colorectal cancer with 1.02 million cases, stomach cancer of 934,000 cases and liver cancer of 626,000 cases. In addition, lung cancer was also reported as the highest cancer deaths with 1.18 million deaths, stomach cancer 700,000 deaths, liver cancer 598,000 deaths, colorectal cancer 529,000 deaths and 411,000 deaths related to breast cancer (Parkin et al. 2005).

Higher cancer incidence in developed regions is most likely attributed to the lifestyle and the most common diagnosed cancers being colorectal, breast and prostate (Parkin et al. 2005; Kintzios & Barberaki 2004). However, in developing

regions, commonly diagnosed cancers are liver, stomach and oesophagus. Developed countries show better prognosis than developing countries. Apparently, early detection of cancers and quality treatment both contribute to good prognosis and higher survival rates (Parkin et al. 2005).

1.1.1 Causes of Cancer

The main cause of cancer is still inconclusive; however factors such as exposure to environmental pollution, poor diet, hereditary factors as well as viral infection have been implicated (Kintzios & Barberaki 2004). Nevertheless, in cancers such as breast and prostate which are regulated by hormones, the influence of genetics plays a greater role. The responsible genes include proto-oncogenes and tumour suppressor genes. The proto-oncogenes stimulate cancerous cells growth whereby tumour suppressor genes serve to prevent cells growth (Kintzios & Barberaki 2004).

Under normal setting, balance synchronisation of both genes contribute to normal cell proliferation but due to mutation, the proto-oncogenes cause excessive cells division while tumour suppressor genes are unable to impede the cells division (Kintzios & Barberaki 2004). Examples of tumour suppressor molecules include pRB, p15, p16, p21 and p53; examples of oncogenes include Erb-B, Ki-ras and c-myc (Bergers & Benjamin 2003).

Environmental factors include groups that may pose as carcinogens due to prolong exposure to certain chemical substances, poor diet, ionising radiation and pathogens (Kintzios & Barberaki 2004).

Cancer is also attributed by viruses and bacteria. Infection by viruses and bacteria contributes to 1.2 million cases of malignancies per year (Balkwill & Mantovani 2001; Kuper, Adami & Trichopoulos 2000) often by human papillomaviruses (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV) and Epstein-Barr virus (EBV). These viruses contribute to arising of cervical cancer, hepatocellular carcinoma (HCC), and oral cancer and lymphoproliferative disorders (Karin & Greten 2005). These viruses work by inhibiting the actions of tumour suppressor gene and often cause inflammation (Karin & Greten 2005; Balkwill & Mantovani 2001). It has been shown that inflammation is strongly related to onset of neoplasm (Philip, Rowley & Schreiber 2004). However, a noteworthy fact is that not all microorganisms work by the mentioned mechanisms. *Helicobacter pylori* which is the main cause of gastric cancer, do not carry oncogenes nor does it inhibit the action of tumour suppressor proteins (Karin & Greten 2005).

1.1.2 Cancer Pathology

In the occurrence of cancerous and malignant cells, there are six vital alterations that take place. They are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis as well as tissue invasion and metastasis (Hanahan & Weinberg 2000).

Most cancers are of epithelial origin such as mammary gland, bronchi, colon, prostate and skin. As growth of tumour progress, the surrounding normal host becomes part of the tumour forming a micro-ecosystem (Vanhoecke et al. 2005). The

tumour hosts cells such as fibroblasts, immune cells, platelets and extracellular matrix which also contain regulators for cancer cell growth, differentiation, invasion and survival. The heterogeneity of the tumour highlights the importance of multi-targeted therapy that aims for cancer cells as well as the surrounding normal host tissues (Vanhoecke et al. 2005).

Genetic mutations of cells typically leads to functional alterations (Vanhoecke et al. 2005). Due to the mutations which occur at typically fast rate, the cells proliferate to form group of cells with normal appearance known as hyperplasia. Cells in the state of hyperplasia then undergo further mutation to form abnormal group of cells known as dysplasia. Dysplasia can endure additional mutation leading to tumour formation (Kintzios & Barberaki 2004). Malignant tumours refer to tumour cells which remain localized in its original place while metastasis refers to formation of new tumours via invasion and spreading of cancer cells.

There are two key properties which are vital for cancer cells to remain viable. One of the most important features is the ability to divide infinitely which is non-existent in normal cells. The second characteristic is the ability of the cell to grow without requiring attachment to another tissue or cells (Kintzios & Barberaki 2004). While normal cells adhere to each other and extracellular matrix, cancer cells do not adhere but they have the ability to migrate including invading other tissues and form masses of cells. Though such malignant cells are small in number, they eventually will turn more aggressive over time (Kintzios & Barberaki 2004; Bergers & Benjamin 2003).

There are three mechanistic phases governing carcinogenesis, namely initiation, promotion and progression (Karin & Greten 2005). In tumour initiation which involves stable genomic alterations, chemical or physical carcinogens cause mutation on DNA of cells; thus triggering the activation of oncogenes and or inactivation of tumour suppressor genes. Tumour promotion involves proliferation of genetically altered cells; there is occurrence of clonal expansion of initiated cells which is due to increased cell proliferation and/or reduced cell death (Karin & Greten 2005). Example of tumour growth promoter include cytokines such as interleukin-1 (IL-1), IL-6 and tumour-necrosis factor (Karin & Greten 2005; Harris 2002).

In cancerous cells, activation of signal transduction pathways in turn triggers activation of membrane bound receptors by autocrine, paracrine and endocrine growth factors. Upon activation of these key factors, cancerous cells are able to proliferate and grow. In contrast to normal cells, signal transduction pathways in cancer cells often gets mutated or overexpressed which leads to activation of elements such as tyrosine kinases growth factor receptor (Lobbezoo, Giaccone & van Kalken 2003). Cancerous cells have the ability to produce their own growth signals with minimal dependence on stimulation from normal tissues environment (Hanahan & Weinberg 2000). Overexpression of growth receptors may elevate the responsiveness of the cancer cells such as overexpression of HER/neu receptor in stomach and mammary carcinomas (Slamon et al. 1987). Expression of signal transduction pathways in tumour cells encouraged the search for drug molecules to act on specific proteins in the pathways. These molecules have been referred to as signal transduction modulators (STMs). Such molecules interact within the pathways by blocking cell surface receptors, impeding the tyrosine kinases growth factor

receptor, or elicit inhibition on mitogen-activated protein kinases. Example of STMs are the FDA approved anti-cancer drugs; trastuzumab and imatinib (Lobbezoo, Giaccone & van Kalken 2003).

1.2 Angiogenesis and Cancer

Angiogenesis is the formation of new blood vessels sprouting from existing vascularisation (Kusaka et al. 1991; Folkman 1990; Rosen 2002). The process can also occur during oxygen and nutrient deprivation in normal tissues and arise as a result of metabolic tissues, new organ and embryogenesis development, wound healing and reproductive functions. In the early 1970s it was noted that solid tumours appear to be highly vascularised (Folkman 1971).

It was then established that all solid tumours are dependent on the angiogenesis process in order for the tumour to grow larger and spread to other parts of the body (Folkman 1990; Folkman 1971). The process also takes place in some pathological conditions such as inflammatory diseases such as rheumatoid arthritis, atherosclerosis, proliferative retinopathies, psoriasis as well as cancers (Rosen 2002).

1.2.1 The Progression of Angiogenesis Cascade

The angiogenesis process is constructed by a series of sequential steps (Eccles et al. 2009; Auerbach et al. 2003). Firstly, the vascular basement membrane of existing blood vessels as well as extracellular matrix are both degraded in order to permit migration of underlying endothelial cells into the perivascular space towards chemotactic angiogenic stimuli (Eccles, Box & Court 2005). The endothelial cells

then start to proliferate leading to massive sprouting enhancing migration towards the perivascular space, to form migration column (Fischer, Schneider & Carmeliet 2006).

The migration columns then direct cells to differentiation whereby the endothelial cells change shape and begin to adhere to each other and cells get organised into forming lumen-capillary tubes with tight junctions and deposition of new basement membrane (Eccles, Box & Court 2005). Endothelial cells within the vascular wall that constantly undergoes proliferation result in larger diameter of the blood vessel. Thus, it attracts perivascular cells which in turn produce a vascular basal lamina around the new blood vessels (Bergers & Benjamin 2003; Mojzis et al. 2008; Fischer, Schneider & Carmeliet 2006). The angiogenesis process is as shown in Figure 1.1.

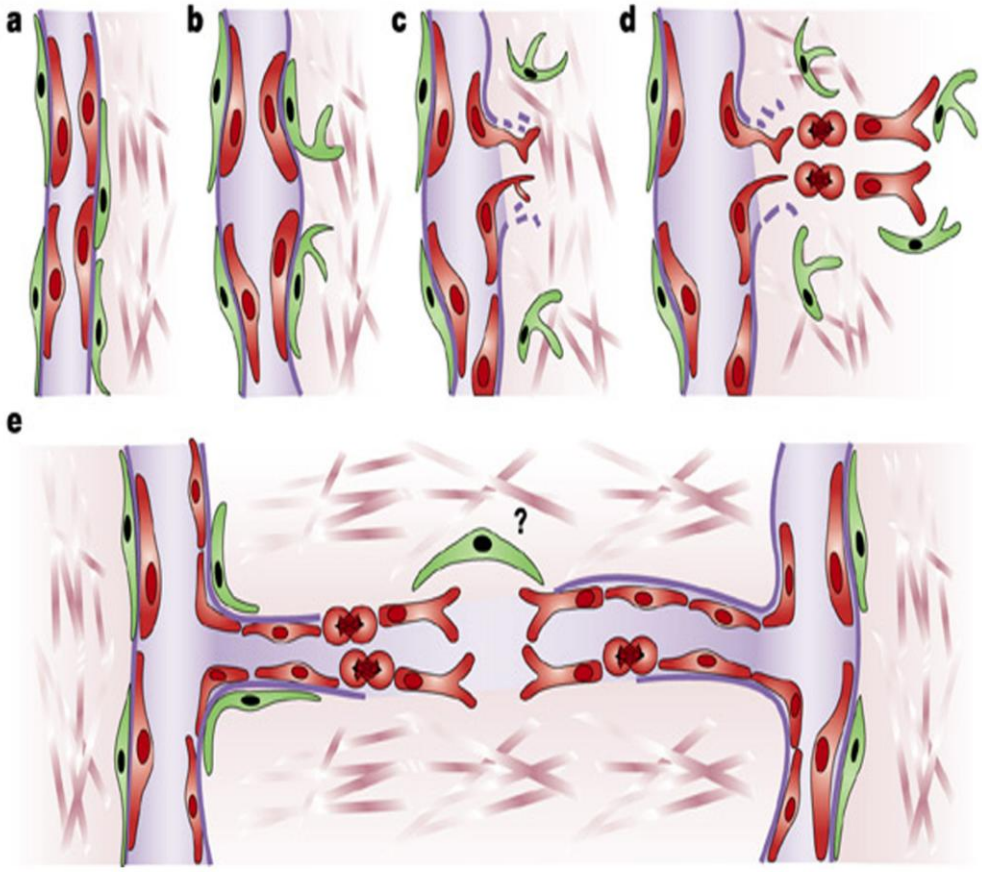

Figure 1.1 The angiogenesis cascade. (a) Blood vessels emerge from existing vasculature, (b) pericytes (in green) detach, blood vessels dilate before basement membrane and extracellular matrix gets degraded (c) which allows endothelial cells to migrate into perivascular space towards angiogenic stimuli, (d) then endothelial cells proliferate, following one another, lead by pericytes, (e) endothelial cells adhere to each other forming a lumen; formation of basement membrane and attachment of pericytes. The sprouting of blood vessels will enable formation of new circulatory systems; however, limited information is present on this fusion mechanism. Adapted from (Bergers & Benjamin 2003).

1.2.2 Role of Angiogenesis Inhibitors in Cancer Therapy

1.2.2.1 Current Methods in Cancer Therapy

In treating cancers, there are two methods which are commonly used. Conventional treatment which includes surgery, radiotherapy and chemotherapy (Kintzios & Barberaki 2004). Surgery is the preferred method of cancer therapy especially when the cancerous tissue is still localised and has not metastasized. However, this method may cause scarring and also injure healthy organs. Furthermore remnants of neoplastic tissues may still be present (Kintzios & Barberaki 2004). In radiation therapy, the cancerous cells are exposed to ionising waves which causes DNA fragmentation leading to apoptotic cell death. However, the presence of radiosensitive cancer cells often leads to treatment failure. Then, immunotherapy and inhibition of angiogenesis, are classified as advanced cancer treatment methods (Kintzios & Barberaki 2004; Pesenti et al. 1992).

Chemotherapy employs cytotoxic agents that either targets the DNA of cancer cells leading to their damage or specific metabolic pathways that are crucial for the cell survival. Treatment of chemotherapy can be generalised into various groups namely anti-metabolites, alkylating agents, topoisomerase inhibitors, antibiotics and anthracyclines (Kintzios & Barberaki 2004). Since cancerous cells have the ability to divide infinitely, they require continuous synthesis of genetic material (DNA). As such, chemotherapy agents target DNA in the effort to impede the cancerous cells' growth (Siddik 2005). Chemotherapy agents kill cancerous cells by interrupting DNA and its replication (Alakananda & Soumya 2011) which inhibit DNA synthesis, hence affecting RNA and proteins productions (Siddik 2005).

Among all chemotherapy drugs, alkylating agents have been identified as the most effective anti-cancer agent used (Alakananda & Soumya 2011). Alkylating agent refers to compounds which can replace hydrogen atom in another molecule by an alkyl radical through electrophilic attack (Warwick 1963). Any distortions and unwinding in DNA caused by interaction between the DNA with alkylating agents will affect the cell cycle development, thereby blocking new DNA replications on the damaged DNA template or prevent damaged chromosomes to be passed on to daughter cells. The action exerted by alkylating agents however often cause side effects due to poor selectivity (Siddik 2005).

There are five classes of alkylating agents; nitrogen mustards, aziridines, alkyl sulphonates, nitrosoureas and mechanistically distinct platinum-containing drugs. Generally, alkylating agents due to their chemical reactivity, form covalent linkages at nucleophilic sites on different DNA bases to induce cross-linkages. While traditional alkylators interact with DNA by guanine bases, the platinum-containing drug form covalent bonding between adenine and or guanine bases via the platinum atom (Siddik 2005). The platinum-containing drug is considered as one example of effective anti-cancer treatment due to its positive response either as single drug or in combination with other standard chemotherapy regimens (Siddik 2005; Alakananda & Soumya 2011). Such example centres on cisplatin. The anti-tumour effect exerted by cisplatin is contributed by its interaction with chromosomal DNA, which causes DNA damage. Like other chemotherapeutic drugs, cisplatin may likely induce apoptosis, occurring via extrinsic or intrinsic pathways (Alakananda & Soumya 2011).

Recent arsenals in cancer therapy are the monoclonal antibodies which target key components of cancer cell receptors and cytokines (Ferrara, Hillan & Novotny 2005; Oldham 1983). In treating cancers, the lack of specificity of the drug to cancer cells is one of the major drawbacks. However, since the introduction of monoclonal antibodies which involves selection of single cells and clonal expansion of a single hybrid between antibodies-forming cell and myeloma cell (Oldham 1983; Hudis 2007), there have been clinically-proven positive responses in patients suffering from metastatic breast (Slamon et al. 2001) and colorectal cancer (Ferrara, Hillan & Novotny 2005).

The mechanisms by which monoclonal antibodies work to affect the tumour cells vary according to each type. Most monoclonal antibodies interact with the immune system through antibody-dependent cellular cytotoxicity (ADCC) which occurs as the antibodies bind to antigens on tumour cells and antibody Fc domains connects with Fc receptors (FcR) on the immune effector cells' surface. The Fc-receptors interactions have been shown to cause substantial anti-tumour activity (Adams & Weiner 2005). Example of such monoclonal antibody is the FDA-approved trastuzumab (Herceptin, Genentech), the first humanised monoclonal antibody which targets HER2 gene in metastatic breast cancer patients (Slamon et al. 2001). The antibody has shown significant survival percentage in patients administered with the antibody alone or in combination with standard chemotherapy (Slamon et al. 2001; Baselga & Albanell 2001).

Monoclonal antibody may also interact with the immune system through complement-dependent cytotoxicity (CDC); binding of monoclonal antibodies to

antigen on the cell surface causes exposure of binding sites on the antibodies which lead to discharge of chemotactic factors and formation of membrane attack complex (Adams & Weiner 2005). Example of such monoclonal antibody is the murine-human chimeric rituximab (Rituxan, Biogen Idec/Genentech) targeting CD20 antigen in treating lymphomas (Adams & Weiner 2005).

Apart from such mechanisms, targeting ligands initiating signalling through receptors has also become of great interest. An example of a monoclonal antibody depicting such action is bevacizumab (Avastin, Genentech/Roche) which primarily targets to block the binding of VEGF-A (an isoform of VEGF) to their receptors on the vascular endothelium (Ferrara, Hillan & Novotny 2005). As VEGF has been identified as the responsible stimulator for new blood vessels growth, thus incorporating bevacizumab in standard chemotherapy regimen for metastatic colorectal cancer has shown improved response rates and survival (Ferrara, Hillan & Novotny 2005; Adams & Weiner 2005; Mukherji 2010).

Endothelial cells in tumour bed tend to be more susceptible to cytotoxic agents due to their high proliferation rate. In addition, in comparison to cancerous cells, endothelial cells are genetically stable as they do not undergo mutations such as p53 mutation and hence prone to apoptotic effects of the cytotoxic agents. Thus, these features of endothelial cells make a compelling target for anti-angiogenesis treatment (Folkman 2003). As such, cytotoxic agents pose as candidate as anti-angiogenic agent on top of their potent activity in causing death of cancerous cells. Paclitaxel, an anti-cancer drug has been shown to inhibit proliferation of endothelial cells as well as cells migration and invasiveness dose-dependently in both *in vitro* and *in vivo*.

Cytotoxic agents cause apoptosis on endothelial cells which then direct apoptosis on cancerous cells thus causing the inhibition of cancerous cells (Folkman 2003).

In demonstrating cytotoxic drug as anti-angiogenic agent, it is best viewed by an alternative administration to maximum tolerated dose (MTD) known as metronomic therapy (Browder et al. 2000; Kerbel & Kamen 2004). Conventional chemotherapy is usually administered at MTD which then need breaks between treatments. During the breaks, endothelial cells were found to proliferate and this contributed to recurrence of the tumour growth (Folkman 2003). Opposing to conventional chemotherapy which suppressed tumour growth but found recurrence of tumour growth leading to drug resistant and cells deaths; metronomic anti-angiogenic scheduling therapy has shown that the drug inhibited significant tumour growth (Kerbel & Kamen 2004). Addition of pure anti-angiogenesis inhibitor with the initial chemotherapy showed complete deterioration of the tumour and high percentage of survival (Browder et al. 2000; Folkman 2003). This has since illustrated the endothelial-dependent on cytotoxic agent and shown potencies of cytotoxic agent as anti-angiogenic agent (Folkman 2003).

Since the introduction of the hypothesis focusing on tumour is angiogenesis-dependent by Folkman in 1971 (Folkman 1971), massive research and development on angiogenesis as well as studies on its pro- and anti-molecules has sparked interest of researchers in angiogenesis-related field worldwide. Extensive studies have been carried out to develop therapeutic strategies such as in stimulating revascularisation of ischaemic tissues as well as development of anti-angiogenesis agent to be used in diseases such as cancers and inflammation (Fischer, Schneider & Carmeliet 2006).

Examples of positive outcome spring forth from anti-angiogenic studies is the FDA-approved bevacizumab (Avastin, Genentech/Roche) for colorectal, breast and lung cancer treatment in combination with conventional chemotherapy. Avastin works by blocking the VEGF expression, thus inhibiting angiogenesis (Fischer, Schneider & Carmeliet 2006; Ferrara, Hillan & Novotny 2005).

Conventional chemotherapy drug is usually administered at maximum dose, which initially causes inhibition of cancerous cells. However, drug-free interval following treatment cause massive proliferation of the endothelial cells that hosts the cancerous cells. Thus, recurrence of tumour growth resumes and speeds up to cause death due to drug-resistance of the tumour. Anti-angiogenic treatment however overcomes this setback by administering low doses without drug-free interval, hence the tumours are significantly inhibited and contributes to higher percentage of survival (Browder et al. 2000).

Anti-angiogenesis has the advantage in succeeding conventional chemotherapy as administration of drugs must cross microvascular endothelium before reaching the tumour cells. Since endothelial are of low probability to mutate and are genetically stable, principally they are readily susceptible to apoptosis effects of the drug. Anti-angiogenic drug then targets endothelial cells before acting on tumour cells (Folkman 2003).

Although given all the advantages of anti-angiogenesis treatment, some disadvantages may emerge. Clinical trials of pro-angiogenic molecules of VEGF and FGF failed to meet initial expected results; responsibilities of these unexpected outcome were pointed to poor delivery strategies and the uncontrolled regulation of

functional vessel growth (Fischer, Schneider & Carmeliet 2006). Then, it is speculated that long duration of conventional chemotherapy combined with anti-angiogenic agent may pose adverse toxic effect (Mukherjee et al. 2004). Nevertheless, the toxicity effect of anti-angiogenic agent can be avoided by adopting promising approach such as biologically active dose that will model as acceptable toxicity profiles (Mukherjee et al. 2004; Mendel et al. 2003). Anti-angiogenic agent may also play a role in normal growth of humans as well as possibilities in disturbing pregnancies as shown by studies on teratogenic drug. However, these drawbacks can be avoided so long that the necessary angiogenesis process are not disrupted (D'Amato et al. 1994).

In the advancement of anti-angiogenesis therapy, therapeutics agents targeting to directly attack tumour blood vessels rather than to inhibit the tumour vascularisation has emerged as one of the latest approach (Frankel et al. 2011). Such approach utilises antagonist effect against expression of some receptors on the endothelial cells lining the tumour vasculature which generates overexpression of tumour endothelial markers (TEMs) such as TEM-8 and capillary morphogenesis gene-2 (CMG-2) (Cryan & Rogers 2011) which are known receptors for mediating access of anthrax toxin into host cells (Werner, Kowalczyk & Faundez 2006) and are highly specific to tumour angiogenesis (Maurya et al. 2011). Since the expression of TEMs level up during the cascades of angiogenesis, this points to possibilities in selectively targeting the tumour endothelium as an approach of anti-angiogenic therapy (Cryan & Rogers 2011; Werner, Kowalczyk & Faundez 2006). Previous studies showed anti-angiogenic potential of CMG-2 and TEM-8 whereby reduction

of their expressions inhibited endothelial proliferation, migration and differentiation (Reeves et al. 2009; Cryan & Rogers 2011).

1.2.2.2 Angiogenic Switch

As initially proposed by Folkman, tumour growth and metastasis are angiogenesis-dependent (Folkman 1971). Thus targeting angiogenesis inhibition may well contribute to blocking of tumour growth. As such, the proposed idea has since generated the search for pro- and anti-angiogenic molecules (Carmeliet & Jain 2000).

In every normal tissue, physiological changes occur as a result of balance between pro- and anti-angiogenic factors. In response to the physiological stimuli, endothelial cells react by its dividing ability. Such mechanism is corresponding to wound healing. However, the physiological stimuli may also become extremely excessive which causes imbalance between stimulators and inhibitors, thus resulting in an angiogenesis switch (Mojzis et al. 2008). The switch is "off" upon balancing between stimulators and inhibitors molecules and "on" when the stimulators outweighs the inhibitors (Carmeliet & Jain 2000). Table 1.1 depicts some stimulators and inhibitors responsible for the angiogenic switch.

The signals triggering the switch include environmental and genetic changes. Such changes are hypoxia, pH variation, metabolic stress, cytokines from inflammatory response as well as genetic mutations potentiated by up-regulating of oncogenes such as Src and Ras and down-regulation of tumour suppressor gene such as p53 (Pang & Poon 2006; Carmeliet & Jain 2000). As a consequence, the switch

gives rise to various disorders such as cancer, infectious diseases, atherosclerosis, arthritis, diabetes, obesity and inflammation diseases (Mojzis et al. 2008).

The introduction of oncogenes into tumours has been shown to rapidly cause the angiogenic switch. This is supported by the observation of transfected human non-angiogenic osteosarcoma with Ras oncogene had led to neovascularisation in two weeks (Folkman 2003). Activity of some hormones such as androgen, progesterone and oestrogens may also promote angiogenesis through carcinogenesis and tumour progression in hormone-dependent cancers such as prostate and breast cancers (Pang & Poon 2006). Thus, it is noteworthy to acknowledge the contributions of the factors in inducing the angiogenic switch are tumour-type dependent. Their contributions are also likely to change with the tumour growth, progression and degeneration (Carmeliet & Jain 2000).

Table 1.1 Examples of some stimulators and inhibitors of angiogenesis

Stimulators	Function	Inhibitors	Function
VEGF	Stimulate angiogenesis and vasculogenesis, induces permeability and leukocyte adhesion	Angiostatin and related plasminogen	Suppress tumour angiogenesis
TGF-β1, endoglin, TGF-β receptors	Stimulate extracellular matrix production	Endostatin	Inhibit endothelial cell survival and migration
Plasminogen activators, MMPs	Remodel matrix, release and activate growth factors	IFN-α, -β, -γ; IL-4, -12, -18	Inhibit endothelial migration and downregulation of bFGF
PDGF-BB and receptors	Recruit smooth muscle cells	Prothrombin	Suppress endothelial growth
FGF, HGF, MCP-1	Stimulate angiogenesis/arteriogenesis	Platelet factor-4	Inhibit binding of bFGF and VEGF

1.2.2.3 VEGF and Its Role in Angiogenesis

Many proteins are associated with regulating the process of angiogenesis. Vascular endothelial growth factor (VEGF) is a protein that stimulates vasculogenesis and angiogenesis (Kondo et al. 2002). VEGF is also known as VEGF-A. The VEGF family, in addition to VEGF-A also comprise of VEGF-B, -C and -D and placental growth factor (PGF) (Ferrara, Hillan & Novotny 2005; Rakic et al. 2003). Regulatory responses of PIGF and its receptor Flt1 (also known as VEGF-R1) on angiogenesis cascade causes loss of VEGF-R1 thwarted angiogenic process as well as vascular leakage in cancer, ischaemia and wound healing (Rakic et al. 2003).

VEGF which is a secreted angiogenic mitogen targeting specifically vascular endothelial cells has been identified as a potent angiogenic factor (Breier et al. 1992). Normally, VEGF helps to stabilise oxygen supply when normal circulation is not enough. VEGF is also responsible in the formation of new blood vessels (Kondo et al. 2002). Apart from stimulating endothelial cell growth and differentiation, VEGF is also noted for its capability in inducing vascular permeability and in attracting monocytes thus enhancing their migration *in vitro* (Breier et al. 1992). However, overexpression of VEGF contributes to diseases such as cancers. Typically, cancer tumour cannot grow without adequate blood supply, so in the case of overexpression of VEGF, the tumour will grow and metastasize (Kondo et al. 2002).

1.2.2.4 Role of Hypoxia in Angiogenesis

Hypoxia is defined as insufficient oxygen level which is needed in maintaining normal cellular functions (Harris 2002). Hypoxic conditions can trigger angiogenesis (Bergers & Benjamin 2003). Hypoxia takes place during acute and chronic vascular disease, pulmonary disease as well as cancers. Acute hypoxia is when formation of new blood vessels of the tumours is abnormal causing insufficient and inefficient blood flow and therefore the needed oxygen supply. Chronic hypoxia on the other hand occurs when the oxygen level is not sufficient to accommodate the massive formation of new blood vessels from uncontrolled proliferation (Harris 2002).

Hypoxia induces transcription programme leading to aggressive tumour phenotype. Hypoxic tumours occur when the new blood vessels formed are abnormal, causing deprivation of blood flow. Hypoxia is toxic to both normal and cancerous cells but under genetic changes, these cells are able to survive and thus proliferation continues (Bergers & Benjamin 2003). Their proliferation thus cause the cells to become more invasive and lead to emergence of malignant phenotype of tumours (Harris 2002).

Hypoxia has long been regarded as one of the key features contributing to tumour, which also makes the cells to become aggressively resistant to apoptosis as well as to conventional cancer treatment such as radiation and chemotherapy (Melillo 2006). Due to hypoxic conditions, there arise several biological responses which are alteration of aerobic to anaerobic metabolism, induction of erythropoietin (EPO) in renal cells, induction of tyrosine hydroxylase synthesis in neural cells and production of growth factor that stimulates angiogenesis (Harris 2002).

Transcription factor hypoxia-inducible factor 1 (HIF-1) is the responsible key player in stimulating the transcriptional response of cells to insufficient oxygen supply (Melillo 2006). HIF-1 is made up of hypoxic factor (HIF-1α) and constitutively expressed aryl hydrocarbon receptor nuclear translocator, ARNT (also known as HIF-1β) composing a heterodimer. Adhering of HIF-1 in the absence of oxygen to hypoxia-response elements such as DNA triggers expression of several pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-3 (FGF) and platelet-derived growth factor-B (PDGF) (Harris 2002). HIF-1 is essential for normal embryogenesis and tumourigenesis (Ryan, Lo & Johnson 1998).

Genes induced by hypoxia regulate several biological processes such as cell proliferation, angiogenesis, metabolism, apoptosis, immortalisation and migration. Production of growth factors such as transforming growth factor-β (TGF-β) and PDGF upon induction by hypoxia, promote cell proliferation (Bergers & Benjamin 2003; Harris 2002). The imperative effect of HIF-1α is prominent, as its deletion may inhibit cell growth and thus angiogenesis. Furthermore, activation of VEGF transcription and one of its receptors, VEGF receptor 1 (VEGF-R1) directly regulates endothelial cell growth proliferation and thus blood vessels formation. Activation of HIF-1 also cause lower expression of anti-angiogenic proteins such as thrombospondin-1 and -2 (Harris 2002).

1.3 Plants with High Antioxidant Property and Their Anti-cancer Property

For centuries, humans have been utilising natural products particularly plants in treating various ailments. In relieving certain illnesses, combination of medicinal herbs is often adopted. In many parts of the world, this traditional medication system is still much appreciated and practiced; sometimes it is the preferred medication option (Cai et al. 2004). Plants and natural products have such immense effect in treating varieties of diseases such as cancers, in fact around 74% of anti-cancer drugs used today originates from natural products (Lopes et al. 2009).

Plants consist of various phytochemical groups which have valuable pharmaceutical applications. Secondary metabolites which refer to compounds consisting of various chemical groups such as phenolics, flavonoids, alkaloids, terpenoids, glycosides, lipids, organic acids and aromatic compounds are among the active phytochemicals occurring abundantly in plants (Kintzios & Barberaki 2004).

Secondary metabolites have the ability to function as protective agents against pathogens such as insects, fungi and bacteria or as growth regulator molecules such as hormone-like substances stimulating or inhibiting cell division and morphogenesis. Given all these great abilities, secondary metabolites stand as potential anti-cancer drugs as they are able to affect cancerous cells directly or stimulate the tumour development or direct inhibition of cells (Kintzios & Barberaki 2004). Examples of some anti-cancer drugs of natural origin are paclitaxel from *Taxus brevifolia* Nutt, vinblastine and vincristine from *Catharanthus roseus* and camptothecin from *Camptotheca acuminata* (Newman, Cragg & Snader 2003).

Extensive studies have been conducted to assess the role of oxidative stress and hence the use of antioxidants in the prevention of many disease processes that occurs in cancer, inflammation, atherosclerosis and age related illnesses (Fernández-Pachón et al. 2004; Chiang, Lo & Lu 1994; Cos et al. 1998). This has since encouraged the search for new natural antioxidants (Barreto et al. 2008). In the initial stage of such diseases, oxidative stress plays a major role. Reactive oxygen species (ROS) which is formed during normal metabolism have the capacity to damage proteins, lipids or DNA. Endogenous antioxidant defense architecture exists and is preliminarily responsible for preventing these damages. Nonetheless, the power of this endogenous system may not be adequate to combat the excessive oxidative damage, therefore it is imperative to have an additional supplement of external antioxidants via dietary source (Fernández-Pachón et al. 2004). Previous studies have also suggested that dietary consumption with high phenolics content may able to reduce the incidence rates of fatal diseases such as cancer and cardiovascular diseases (Kris-Etherton et al. 2002; Ramarathnam et al. 1995).

To date, there have been extensive studies on natural products compounds and extracts that show potent anti-angiogenic activity, in conjunction to having good antioxidant abilities (Cao & Cao 1999; Lamy, Gingras & Béliveau 2002; Lamy et al. 2006). Green tea catechins and crude green tea extracts were found to inhibit both breast cancer cells and proliferation of endothelial cells *in vitro*. The green tea extracts has shown significant reduction in VEGF peptide expression in both cells with dose-dependent effect (Sartippour et al. 2002).

Prominent components of green tea, epigallocatechin-3-gallate (EGCG) as well as the crude tea extract itself were found to show specific inhibition on endothelial cells growth and inhibition of new microvessels growth in chorioallantoic membrane assay. The tea extract also showed prevention of corneal angiogenesis which had been pre-stimulated with VEGF (Cao & Cao 1999). EGCG also significantly suppressed hypoxia and serum-induced HIF-1α protein build up in human cervical carcinoma (HeLa) and hepatoma (Hep G2) cancer cells. Inhibition of HIF-1α caused significant reduction in VEGF expression in mRNA and proteins levels (Zhang et al. 2006).

Anthocyanidins, a type of phenolic compound has been found to cause downstream signalling of VEGF-R2; it also inhibits the formation of capillary-like tubular structures *in vitro* as well as inhibition of basic fibroblast growth factor-induced vessel formation in the mouse matrigel plug assay (Lamy et al. 2006). Silibinin, a type of flavonoid showed suppression on tumour xenograft model via reduction in the neovascularisation leading to increased apoptosis. Silibinin also inhibited migration in HUVECs. Induction of apoptosis was observed in both caspase-dependent and independent mechanisms (Singh et al. 2004). Quercetin, a major flavonoid constituent has been reviewed extensively with antioxidant, anti-inflammatory, cardioprotective effects as well as anti-cancer activity. It also exhibited significant inhibition on angiogenesis cascades such as proliferation, migration and tube formation of human microvascular dermal endothelial cells dose-dependently (Mojzis et al. 2008).