# CYTOTOXIC ACTIVITY AND MECHANISMS OF ACTION OF *CRINUM ASIATICUM* EXTRACTS ON VARIOUS CANCER CELL LINES

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**Thesis submitted in fulfilment of the requirements**

**for the degree of**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| AP-1 | Activator protein-1 |
| APC | Adenomatous polyposis coli |
| ATCC | American type culture collections |
| BA | Betulinic acid |
| BSA | Bovine serum albumin |
| CO2 | Carbon dioxide |
| ddH2O | Deionised distilled water |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DNA-RC | DNA replication complex |
| DR | Death receptor |
| EAhy 926 | Hybrid endothelial cell line |
| ECGS | Endothelial cell growth supplement |
| EDRF | Endothelium derived relaxing factor |
| ELK 1/SRF | ETS Like gene 1/spectral repeat finder |
| eNOS | Endothelial nitric oxide synthase |
| ESI | Electrospray Ion |
| FADD | Fas-associated protein with death domain |
| FAK | Focal adhesion kinase |
| FDA | Food and Drug Administration |
| FGF | Fibroblast growth factor |
| g | Gram |
| GTP | Guanosine triphosphate |
| h | Hour |
| HCT-116 | Colon cancer cell line |
| Hep-2 | Laryngeal cancer cell line |
| HIF-1 | Hypoxia inducible factor one |
| HIFCS | Heat inactivated foetal calf serum |
| HRP | Horseradish Peroxidase |
| $IC_{50}$ | Inhibitory concentration of 50% |
| IFN | Interferon |

| | |
|---|---|
| IL | Interleukin |
| KBr | Potassium bromide |
| GC-MS | Gas chromatography mass spectrometry |
| MAPK | Mitogen activated protein kinase |
| MAPK/ERK | MAPK-Extra cellular signal regulated enzyme kinase |
| MAPK/JNC | MAPK-C-Jun amino terminal kinase |
| MCF-7 | Breast cancer cell line |
| min | Minute |
| ml | Millilitre |
| mm | Millimetre |
| mM | Millimolar |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger Ribonucleic acid xiv |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide |
| Myc/Max | A signal transduction pathway |
| NaCl | Sodium chloride |
| NF-κB | Nuclear factor kappa B |
| NIH | National Institute of Health |
| Notch | A signal transduction pathway |
| NO | Nitric oxide |
| N2 | Nitrogen gas |
| PARP | Poly ADP ribose polymerase |
| PBS | Phosphate buffer saline |
| PDGF | Platelet derived growth factor |
| PE | Plating efficiency |
| pg/ml | Picogram per millilitre |
| PRb | Retinoblastoma tumour suppressor protein |
| pRb-E2F | A signal transduction pathway |
| Psi | Pound per square inch |
| P1GF | Placental growth factor |
| P-338 | Murine lymphocytic leukaemia |
| RB | Retinoblastoma |
| RF | Resonance Frequency |
| ROCK-1 | Rho-associated protein kinase 1 |

| | |
|---|---|
| SF | Survival fraction |
| SMAD | Mother against decapentaplegic |
| STM | signal transduction modulators |
| TCF/LEF | T-cell factor/lymphoid enhancer factor |
| TGF-β | Tumour growth factor beta |
| TMS | Tetramethylsilane |
| TNF 1 | Tumour necrosis factor 1 |
| TPA | 12-O-tetradecanoyl phorbol-13-acetate |
| v/v | Volume per volume |
| VEGF | Vascular endothelial growth factor |
| VPP | Volts, Peak-to-Peak |
| WHO | World Health Organization |
| Wnt | wingless-int , a signal transduction pathway |
| w/v | Weight per volume |
| µg/ml | Microgram / millilitre |
| µl | Microlitre |

**AKTIVITI SITOTOKSIK DAN MEKANISME-MEKANISME TINDAKAN EKSTRAK-EKSTRAK *CRINUM ASIATICUM* KE ATAS PELBAGAI TITISAN SEL KANSER**

## ABSTRAK

*Crinum asiaticum* yang dikenali oleh penduduk tempatan sebagai tembaga suasa, digunakan dalam perubatan tradisional, untuk merawat inflamasi/bengkak dan tumor. Beberapa kandungan kimianya telah diasingkan dan didapati mempunyai kesan anti-tumor, termasuk likorin. Tujuan penyelidikan ini ialah untuk mengkaji aktiviti sitotoksik (sitotoksisiti) dan mekanisme tindakan, tumbuhan spesis Malaysia ini, ke atas pelbagai titisan sel kanser. Ekstrak-ekstrak methanol akar, ubi, batang dan daun, difraksikan lagi dengan menggunakan heksana, dietil eter, butanol dan air suling. Kesemua ekstrak diuji dengan menggunakan assai MTT, ke atas tiga titisan sel kanser manusia (3 daripada 4 kanser yang membentuk 50% kematian akibat kanser): Hep-2 (kanser larynx), HCT-116 (tumor kolorektal manusia) dan MCF-7 (kanser payudara); dan dua titisan sel normal: Vero (medula ginjal monyet hijau Afrika) dan EAhy-926 (hibrid sel-sel endotelial dan kanser paru-paru manusia). Ujian lanjutan telah dijalankan untuk membandingkan sitotoksisiti fraksi-fraksi ekstrak ke atas sel-sel Vero dan Hep-2. Kesan anti-angiogenik juga telah dikaji, menggunakan assai bulatan aorta tikus, migrasi sel dan pengeluaran VEGF manusia dalam sel-sel EAhy-926. Mekanisma tindakan sitotoksisiti kemudian telah dikaji, melalui kajian klonogenisiti, kondensasi nuklear, perubahan potensi membran mitokondria dan kesan-kesan ke atas 10 laluan major kanser iaitu, wnt (TCF/LEF), notch (RBP-Jκ), p53/kerosakan DNA, TGFβ (SMAD2/3/4), putaran sel/pRb-E2F (E2F/DP1), NFκB, myc/Max, hipoksia (HIF1α), MAPK/ERK (Elk-1/SRF) dan MAPK/JNK (AP-1). Ekstrak metanol daun *C. asiaticum* (CALME) menunjukkan

sitotoksisiti yang signifikan ke atas sel Vero, sementara ekstrak-ekstrak metanol lain tidak menunjukkan kesan signifikan. Tiada perbezaan sitotoksisiti yang signifikan di antara pelbagai ekstrak, yang diambil daripada bahagian-bahagian pokok yang berlainan, dan juga, fraksi-fraksi ekstrak**.** CALME menunjukkan kesan sitotoksik aktif ke atas semua sel-sel yg terpilih, kecuali sel Vero (kesan sitotoksik sederhana). Nilai '$IC_{50}$' yang diperolehi adalah di antara 4.15 dan 46.88 µg/ml. Ekstrak juga menunjukkan selektiviti tinggi untuk MCF-7 dan HEP-2, berbanding dengan VERO, dan hampir selektif untuk MCF-7, berbanding dengan EAhy-926. Kajian klonogenisiti ke atas sel-sel MCF-7 menunjukkan CALME adalah sitotoksik, dan bukan sitostatik. Perubahan-perubahan morphologi apoptotik lewat dalam sel juga dikesan; CALME menyebabkan kondensasi DNA dan gangguan potensi membran mitokondria. CALME telah didapati menyebabkan apoptosis dalam sel-sel MCF-7, samada melalui pengawalaturan menurun gene-gene pro-proliferatif **(**hipoksia dan MAPK/JNK), atau melalui pengawalaturan menaik gene-gene perencat tumor **(**p53, TGF-β, pRb-E2F, and NF-κB). Kajian ini mendapati bahawa CALME merencat pertumbuhan saluran darah baru, dan oleh itu, menunjukkan kesan anti-angiogenik. Perencatan migrasi sel dan produksi VEGF oleh ekstrak ini, juga menyokong aktiviti anti-angiogeniknya. Walaubagaimanapun, aktiviti anti-tumor CALME adalah disebabkan oleh sitotoksisiti, bukan aktiviti anti-angiogenik. Keputusan-keputusan spektra mengesahkan kehadiran alkaloid aktif likorin dalam ekstrak daun tempatan, seperti yang terdapat dalam spesis dari India. Keputusan-keputusan kajian ini jelas menunjukkan potensi anti-kanser CALME terhadap kanser payudara.

**CYTOTOXIC ACTIVITY AND MECHANISMS OF ACTION OF *CRINUM ASIATICUM* EXTRACTS ON VARIOUS CANCER CELL LINES**

## ABSTRACT

*Crinum asiaticum,* locally known as 'tembaga suasa', is used traditionally to treat inflammations and tumours. Several chemical constituents had been isolated and found to have anti-tumour property, including lycorine. This study aims to investigate the cytotoxic activity (cytotoxicity) and mechanisms of action of the Malaysian species plant, on various cancer cell lines. The methanol extracts of the root, bulb, stem and leaf were further fractionated using hexane, diethyl ether, butanol and distilled water. All extracts were screened, using MTT assay, against three human cancer cell lines (3 of 4 cancers that form 50% cancer deaths): Hep-2 (laryngeal carcinoma), HCT-116 (human colorectal tumour) and MCF-7 (breast cancer); and two normal cell lines: Vero (African green monkey kidney medulla) and EAhy-926 (hybrid of human endothelial and lung cancer cells). Further tests were performed to compare the cytotoxicity of extract fractions on Vero and Hep-2 cells. The anti-angiogenic effect was also studied, using rat aorta ring assay, migration and human VEGF production in EAhy-926 cells. The mechanisms of cytotoxicity were then studied, by clonogenicity study, determination of nuclear condensation, mitochondrial membrane potential changes, and the effects on 10-major cancer pathways, wnt(TCF/LEF), notch(RBP-Jκ), p53/DNA damage(p53), TGFβ(SMAD2/3/4), cell cycle/pRb-E2F(E2F/DP1), NFκB, myc/Max, hypoxia(HIF1A), MAPK/ERK(Elk-1/SRF) and MAPK/JNK(AP-1). *C. asiaticum* leaf methanol extract (CALME) showed significant cytotoxicity against Vero cells, while the other methanol extracts did not show significant effects. There was no significant difference between the cytotoxicity of extracts taken from different parts

of the plant, and also extract fractions**.** CALME showed active cytotoxicity on all selected cell lines except for Vero (moderately cytotoxic). $IC_{50}$ values obtained ranged from 4.15 to 46.88 µg/ml. The extract also showed high selectivity for MCF-7 and HEP-2, compared to VERO, and marginally selective for MCF-7, compared to EAhy-926. The clonogenicity study on MCF-7 cells indicated CALME to be cytotoxic, and not cytostatic. The late apoptotic morphological changes in cells were also detected; CALME caused DNA condensation and disrupted mitochondrial membrane potential. CALME was found to induce apoptosis in MCF-7 cells, either by down-regulating pro-proliferative genes (hypoxia and MAPK/JNK), or by up-regulating tumour suppressor genes (p53, TGF-β, pRb-E2F, and NF-κB). Thus, the main mechanism of action for cytotoxicity is apoptosis via the mentioned pathways. This study also found that CALME inhibited new blood vessel growth, thus, exhibiting anti-angiogenic effect. The cell migration and VEGF production inhibition by CALME further support its anti-angiogenic activity. However, the anti-tumour activity of CALME is due to cytotoxicity, not anti-angiogenic activity. The spectral results confirmed the presence of the active alkaloid lycorine in the local leaf extract, as found in the Indian species. The results of this study clearly highlight the anti-cancer potential of CALME towards breast cancer.

# CHAPTER 1: INTRODUCTION

## 1.1 Cancer

Billions of dollars have been spent on cancer research and yet we do not understand exactly what cancer is. It has been difficult to develop an accurate definition for cancer. The reputed British oncologist Willis has defined cancer as "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change" (Cotran et al., 1999). Cancer is the abnormal growth of cells in our bodies that can lead to death. It is a group of more than 100 different diseases, which are characterized by uncontrolled cellular growth, local tissue invasion and distant metastasis (Chabner, 2006).

The mechanisms by which cancer arises are incompletely understood. The cancer is assumed to develop from cells in which the typical managing mechanisms of the cells' proliferation and growth have been altered. Recent proofs strengthen the notion of carcinogenesis as a genetically regulated multistage process. The first step in this process is 'initiation' which starts by exposure of cells to carcinogenic substances which lead to genetic damage that, if not repaired, results in irreversible mutations. The mutated cells grow till formation of a colony. The second stage is 'promotion', in which carcinogens or other factors modify the environment, in a way which supports growth of mutated cells over normal cells (Mediana and Fausel, 2008). The next stage is 'transformation' of mutated cells to cancerous cells; about 5 to 20 years may be required for the transition of benign carcinogenic phase to the fully developed malignant stage, where the cancer can be detected clinically. The last stage called 'progression', where further genetic changes take place, leading to increase in the proliferation and metastasis (Weinberg, 1996, Compagni and Christofori, 2000).

### 1.1.1 Cancer Epidemiology

Every year, millions of people are diagnosed with cancer, leading to death. It is estimated that almost 1.7 million new cases of cancer will be diagnosed in 2017. Prostate cancer is the most common cancer among males (19%), followed by lung (14%) and colorectal (9%) cancers. Among females, breast (30%), lung (12%), and colorectal (8%) cancers are the most common (American Cancer Society, 2017).

In Malaysia, cancer was the number 4 killer in the year 2002. However, in the year 2014, it became the number 1 killer (Ministry of Health Malaysia, 2014). Worldwide now, it is also the most lethal among all types of diseases (World Health Organization, 2014).

Lung cancer is by far the leading cause of cancer death among males (27%), followed by colorectal (9%) and prostate (8%) cancers. Among females, lung (25%), breast (14%), and colorectal (8%) cancers are the leading causes of cancer death (American Cancer Society, 2017). Thus, cancers of the lung and bronchus, prostate, and colorectal in men, and cancers of the lung and bronchus, breast, and colorectal in women, form about 50% of cancer deaths among men and women.

The 5-year survival rates have been increased as new drugs and treatment approaches have been introduced; in 1930s, only 20% of cancer patients survived five years or more after treatment. In 1940s the survival rates increased to 25%. During the 1960s, the 5-year survival rate was 33%, and in the 1970s it was 38%. Between years 2000 to 2005, 51% of cancer patients survived for five years or more (Rodriguez and Case, 2005).

However, while the survival rate increases, the incidence and mortality rate also keep on growing (Parkin, 2001). This contradiction between increasing of the 5-years' survival rate and the worldwide death rate due to cancer, can be explained by increased life anticipation, since cancer incidence increases with age (Ries et al., 2000, Jemal et al., 2009).

### 1.1.2 Genetic and Molecular Basis of Cancer

Developing cancer cells select mutations having two basic functions: mutations which increase the activity of the proteins they code for, or mutations which inactivate gene function. The gene whose activity increases by mutation, is called oncogene, while that inactivated by mutation, is called tumour suppressor gene.

Oncogenes are involved in signalling pathways which stimulate proliferation, while human suppressor genes code for proteins which normally act as checkpoints to cell proliferation. These alterations occur by carcinogenic agents like radiation, chemicals or viruses (somatic mutations), or they may be inherited (germ-line mutation) (Croce, 2008, Bertram, 2000, Mediana and Fausel, 2008, Yarbro et al., 2005).

Six major pathways must be activated or inactivated in the genes of normal cells to convert to cancerous cells; these are:

i. development of independence in growth stimulatory signals e.g., activation of a family of oncogenes called human epidermal growth factor receptor (Hanahan and Weinberg, 2000),

ii. development of a refractory state to growth inhibitory signals e.g., mutations in suppressor genes p53 (Feng et al., 2008),

iii. development of resistance to programmed cell death e.g., over expression of *Bcl-2* genes (Kroemer, 1997),

iv. development of an infinite proliferative capacity e.g., over expression of telomeras enzyme (Holt and Shay, 1999),

v. development of angiogenic potential i.e., the capacity to form new blood vessels and capillaries e.g., over expression of VEGF (Folkman, 1995), and

vi. tissue invasion and metastasis e.g., over expression of Myc oncogenes (Kawashima et al., 1988).

### 1.1.3 Cell Death and Apoptosis

Cell death is a key event in biology. Cells die via two main processes; either necrosis or apoptosis (programmed cell death) (Kerr et al., 1972). Apoptosis is firmly regulated by complex molecular signalling systems. Apoptosis plays a key role in development, morphogenesis, tissue remodelling and disposing of aged or damaged cells. The initial definition of apoptosis was morphological: Dying cells exhibit a characteristic pattern of changes, including cytoplasmic shrinkage, active membrane blebbing, chromatin condensation, and, typically, fragmentation into membrane-enclosed vesicles (apoptotic bodies) (Wyllie et al., 1980). In this process the cells activate an intracellular death programme and kill themselves in a controlled way. Because apoptotic cells shrink during this process, they are rapidly digested, thus, there are no leakages of their contents (Raff, 1998).

In contrast, necrosis is a term that describes uncontrolled process in which death takes place after exposure to an acute injury. The cells swell and burst, spilling their content over the surrounding tissue and cause inflammation (Raff, 1998). Any change in apoptosis rate in the body, either increasing or decreasing may cause many diseases. Apoptosis hyper-activation associated with neurodegenerative diseases (e.g., Parkinson's and Alzheimer's syndromes), hematologic diseases (e.g., aplastic anaemia and lymphocytopenia) and disease characterized with tissue damage (e.g., myocardial infarction). On the other hand, increasing cell survival via apoptosis inhibition is related to autoimmunity diseases (e.g., systemic lupus erythematosus) and tumour growths (Chamond et al., 1999).

Studies of tumour growth kinetics showed that changes in cell loss factors, have influence on tumour growth or regression. Further studies revealed a high rate of apoptosis occurrence in tumours treated with cytotoxic agents that regressed after treatment (Kerr et al., 1972).

Apoptosis can be induced through a number of pathways by proteins that control the cell cycle machinery including p53, Wnt, hypoxia, NF-κB, Notch and MAPKs pathways

(Ghobrial et al., 2005). Any defects in these regulatory pathways have been related to many malignancies (Kaufmann and Hengartner, 2001).

### 1.1.4 Apoptosis Pathways

Apoptotic signalling events can be divided into two major pathways based on the mechanism of initiation: the intrinsic pathway which mainly depends on mitochondrial changes, and the extrinsic pathway which is activated via death receptors. Although different molecules take part in the core machinery of both apoptosis signalling pathways, a crosstalk exists at multiple levels (Ghobrial et al., 2005).

### 1.1.5 Signal Transduction Pathways in Cancer:

Cancer cells have the ability to change the surrounding environment in a way that will assist them to grow and proliferate. They respond to any internal or external circumstances by increasing or decreasing the expression of proteins which can adjust the situations in favour of increasing the proliferative, invasive and metastatic properties (Hanahan and Weinberg, 2000). The reciprocal communications between the external or internal circumstances and protein expression level take place via activation of a cascade of intracellular biochemical reactions which is also called signal transduction pathways (Lobbezoo et al., 2003). Each pathway starts with ligation of extracellular receptors. The receptor activation is translated into biological response by activation of proteins (transcriptional factors) which then translocate into the nucleus and bind with the DNA in specific binding sites (promoters) and trigger the transcription of mRNAs which later translated to proteins (Eccleston and Dhand, 2006).

Oncogenic gene mutations results in a constitutive activation of signal transduction elements, simulating a condition of permanent activation of the receptor, even in the absence of the relevant growth factor (Hanahan and Folkman, 1996). Wnt, Notch, TGF-β, Myc/Max, Hypoxia, MAPK pathways were reported to be hyper-activated in cancerous cells (Clevers,

2004, Miyazawa et al., 2002, Fang and Richardson, 2005, Soucek et al., 2008, van Es and Clevers, 2005a).

On the other hand, mutations in tumour suppressor genes lead to deactivation of some pathways which may serve as checkpoints of cell proliferation such as p53 (Feng et al., 2008). These pathways can be targeted with signal transduction modulators (STMs) in order to treat cancer. The STMs can modulate the pathway activity at many levels such as blocking cell surface receptors, blocking the mediators between extracellular signals and the transcriptional factor, deactivate the binding between the transcriptional factors with the promoters or inhibiting the effects of further downstream genes (Lobbezoo et al., 2003).

STMs have attracted attention of many researchers. Many STM compounds are being investigated in preclinical studies or in clinical trials. Additionally, there are two approved STM drugs which have been commercially marketed; trastuzumab and imatinib (Lobbezoo et al., 2003).

### 1.1.6 Angiogenesis

Angiogenesis is a process of new blood vessel development. It occurs in the healthy body for healing wounds and for restoring blood flow to tissues after injury or insult. The healthy body controls angiogenesis through a series of "on" and "off" switches. When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the balance is tipped in favour of blood vessel growth. When inhibitors are present in excess of stimulators, angiogenesis is stopped. The normal, healthy body maintains a perfect balance of angiogenesis modulators. In general, angiogenesis is "turned off" by the production of more inhibitors than stimulators. In many serious disease states, the body loses control over angiogenesis (Harris, 1997).

Angiogenesis-dependent diseases result when new blood vessels either grow excessively or insufficiently. Angiogenesis plays an important role in many diseases, such as cancer, rheumatoid arthritis, and diabetic retinopathy (Carmeliet, 2005, Folkman, 1995). Drugs that

modulate, inhibit or stimulate angiogenesis, can be useful to treat a variety of diseases that stem from angiogenesis dysfunction.

In cancer, angiogenesis plays an important role in tumour growth, without which, neoplastic tissues will be unable to expand beyond 1 to 2 $mm^3$ (Folkman and Cotran, 1976). Cancer cells within the tumour can also use the newly formed blood vessels as a port to metastasize to other localities (Weidner et al., 1991). Since the interdependency and a close relationship between angiogenesis, cancer growth and metastasis has been well-established, much efforts have been invested into development or discovery of anti-angiogenic compounds, to treat cancer and a variety of other angiogenic related ailments.

### 1.1.6 (a) The Vascular Endothelial Growth Factor (VEGF)

As the size of the tumour increases, oxygen demand increases, causing a state of hypoxia (Fu et al., 1976). The hypoxic state in the tumour spring forth oxygen free radicals, which in turn activates vascular endothelial growth factor (VEGF) triggering the angiogenesis event (Mukhopadhyay et al., 1995). VEGF (referred to also as VEGF-A) is regarded as a heparin binding angiogenic growth factor exhibiting high specificity for endothelial cells (Gospodarowicz et al., 1989). VEGF is responsible for triggering various steps in the angiogenesis cascade such as proliferation, migration and cell survival (Ferrara, 2002). The tumour regression and inhibition can be achieved by deactivating VEGF activity via neutralizing antibodies or by introduction of dominant negative VEGF receptors (Kim et al., 1993). The VEGF protein and RNA levels are significantly upregulated in most types of cancer. The high concentration of VEGF in cancer patients is associated with poor prognosis (Paley et al., 1997).

### 1.1.6 (b) Angiogenesis Process Cascade

Angiogenesis is a sophisticated multistep process. Increasing of VEGF-165 expression is crucial in initiating the angiogenesis process (Nagy et al., 2003). Angiogenesis starts with

dilatation of blood vessels to increase the permeability to the angiogenesis signals. Then, the pericytes which cover the blood vessels, detach and the vascular basement membrane and extracellular matrix get degraded, which allow for the underlying endothelial cells to migrate into the perivascular space towards chemotactic angiogenic stimuli (Ko et al., 2007). The migrated endothelial cells proliferate, loosely following each other into the perivascular space and form migration columns. Then, the endothelial cells differentiate; cells' shape change in a way that facilitates the cell-cell adherence which then forms a lumen (tube-like structure). Perivascular cells are attracted, and a vascular basal lamina is produced around the newly formed vessels. The details are still vastly obscure for the last stages when vascular sprouts fuse with other sprouts to form loops (Bergers and Benjamin, 2003, Carmeliet, 2005).

### 1.1.6 (c) Cancer is Angiogenesis Dependent

Many studies have confirmed the hypothesis that tumour growth is reliant on neovascularisation process. Any significant increment in tumour size must be in synchrony with increment in the blood supply and blood vessels size (Folkman, 1990). The hypothesis was confirmed by many experimental evidences. In one study, tumours implanted in places where there is no probability of new blood vessels to grow such as the aqueous fluid of the anterior chamber of the eye remained viable, avascular, and limited in size ($<1\ mm^3$). When the cells were implanted on iris vessels, they induced new blood vessels formation which grew hastily reaching 16,000 times their original size within two weeks (Gimbrone et al., 1972). Another evidence that strengthened this hypothesis was the detection of exponential and rapid growth of tumours that were implanted on the chorioallantoic membrane of the chick embryo after blood vessels formation (Knighton et al., 1977). Studies on subcutaneously implanted tumours showed that the blood vessels form approximately 1.5% of the tumour volume; this number represents 400% increase over normal subcutaneous tissue (Thompson et al., 1987).

### 1.1.6 (d) The Angiogenic Switch

It takes between five to twenty years for the transition from benign carcinogenic phase to the fully developed malignant stage where the cancer can be perceived clinically (Mediana and Fausel, 2008). Dormancy stage occurs when tumour cells proliferate but the rate of tumour cell death (apoptosis) counterbalances this proliferation and maintains the tumour mass in a steady state (Ribatti et al., 1997). At this stage, there is a balance between two contrary signals; angiogenesis signals like VEGF (Ferrara et al., 2003) and anti-angiogenesis signals (e.g., endostatin, angiostatin) (Kim et al., 2000). Therefore, the angiogenesis process starts only when the net balance between these contrary signals is tipped in favour of angiogenesis initiation (Hanahan and Weinberg, 2000, Hanahan and Folkman, 1996).

Accordingly, identification and interrupting of the factors and the circumstances which increase the probability of angiogenesis initiation may keep the cancerous cells in the stage of dormancy (Gullino, 1978). The studies showed that angiogenesis process can be triggered by a variety of signals including metabolic stress (e.g., hypoxia or hypoglycaemia), mechanical stress (e.g., pressure generated by proliferating cells), immune/inflammatory response (immune/inflammatory cells that have infiltrated the tissue), and genetic mutations (Hanahan and Weinberg, 2000). These circumstances cause synthesis or release of angiogenic factors such as VEGF (Ribatti, 2009).

### 1.1.6 (e) Hypoxia

Hypoxia is defined as a decrease in the oxygen supply to a level insufficient to maintain cellular function. The cells become hypoxic if it is located too far away from blood vessels. Due to cell proliferation and tumour growth, the cells in the core of tumour, become hypoxic (Carmeliet, 2005). Hypoxic cells are more invasive and metastatic, and more resistant to chemotherapy or radiation (Melillo, 2007).

Recent evidence demonstrated the impact of activation of hypoxia inducible transcription factors (HIFs) in hypoxic cells in angiogenesis process (Zhong et al., 1999). The binding of

HIFs to the DNA, induces expression of several angiogenic factors including VEGF, nitric oxide synthase, platelet-derived growth factor (PDGF), and many others (Ahmed and Bicknell, 2009, Carmeliet, 2005). The critical step in induction of this pathway is the stabilization of the HIFs. The most important two members of HIFs are HIF-1 and HIF-2.

HIF-1 is ubiquitously expressed, while HIF-2 is expressed only in endothelial cells and in the kidney, heart, lungs and small intestine (Wang et al., 1995, Semenza, 2001). HIF-1 complex is a heterodimer consisting of two DNA binding proteins, HIF-1α and HIF-1β. The expression of HIF-1α is tightly regulated by oxygen, while the HIF-1β is expressed constitutively (Bracken et al., 2003, Wang et al., 1995). Under normoxic conditions, HIF-1α is rapidly degraded due to enzymatic prolylhydroxylation. However, under hypoxic conditions the stability and half-life of HIF-1α increased remarkably. Accordingly, HIF-1α dimerizes with HIF-1β. The heterodimer is then translocated to the nucleus and activates the promoter region of target genes (Wang et al., 1995).

As the expression of the chief factor in the angiogenesis, VEGF, and many angiogenic pathways is related directly with the activation of HIF-1, the search for drugs targeting HIF is currently receiving a lot of attention (Semenza, 2003). The notion of targeting HIFs to treat cancer was proven experimentally by a study that showed that tumour growth was reduced significantly in mice implanted with cells infected with a polypeptide which disrupted the binding of HIF-1α to its transcriptional co-activators (Kung et al., 2000). Several approaches targeting tumour hypoxia have been proposed, including prodrugs activated by hypoxia, hypoxia-selective gene therapy and the use of recombinant obligate anaerobic bacteria (Brown and Wilson, 2004). Besides, the mechanism of action for some anti-angiogenic natural compounds such as betulinic acid, Klugine, isocephaeline, emetine and taxol has been confirmed to involve hypoxia pathway inhibition (Karna et al., 2010, Fan et al., 2006, Zhou et al., 2005).

### 1.1.6 (f) Anti-angiogenesis Targets

Agents that suppress or stop neovascularisation often do so by interfering with an essential step in this process, such as: (i) inhibition of endothelial cells activation which may be achieved via inhibition of growth factor signal production, inhibition of receptors production and / or inhibits the binding between signals and receptors, (ii) inhibition of endothelial cells proliferation, (ii) inhibition of endothelial cells migration, (iv) inhibition of endothelial cells differentiation to form a three dimensional tube-like structure and (v) induction of apoptosis in endothelial cells (Zhang and Bicknell, 2001).

### 1.1.6 (g) Pros and Cons of Angiogenesis Treatment

The main difference between the cytotoxic chemotherapy and anti-angiogenic therapy is the target; the chemotherapy agents target tumour cells itself, but antiangiogenic compounds target the endothelial cells. Accordingly, anti-angiogenic compounds gained many advantages over conventional cytotoxic agents; endothelial cells are highly genetically stable with low mutation rate, thus the probability to develop resistance against anti-angiogenesis agents is very low compared to cytotoxic agents (Folkman, 2003). Moreover, little or no toxicity is associated with anti-angiogenic therapy. This advantage is expected as anti-angiogenic compounds target specific immature characteristics of tumour vasculature (Kerbel, 2000).

Another important advantage is that endothelial cells are highly accessible to therapeutic agents, because the endothelial cells are directly exposed to blood borne substances (Schliemann and Neri, 2007). Nevertheless, anti-angiogenic compounds possess some drawbacks; they also interrupt normal physiological neovascularisation process such as pregnancy, wound healing, children growth and also likely to be responsible in inducing amenorrhea (D'Amato et al., 1994). However, many experts strongly believe that these

mentioned threats of the anti-angiogenic drugs are exceptionally low. The anti-angiogenic drug regime can be halted in patients with wounds or utilization of a topical angiogenesis-inducing agent may be employed; so as not to perturb the wound healing progress.

The use of anti-angiogenic agents in young patients has also turned out to be safe given that the major fraction of the angiogenesis process that occurs during development stages has ceased. Therefore, the opposing effects of the drugs are considered to be insignificant. The notion that the anti-angiogenic drugs may thwart pregnancy as well as inducing amenorrhea, are in fact advantageous, considering these factors are not favoured in patients undergoing the anti-angiogenic drug treatment (Zhang and Bicknell, 2001).

### 1.1.7 Natural Products and Cancer Treatment

Some secondary metabolites of plants such as alkaloids, terpenoids and glycosides, serve either as protective agents against various pathogens (e.g., insects, fungi or bacteria) or growth regulatory molecules (e.g., hormone-like substances). As a result, secondary metabolites can serve as potential anti-cancer drugs, either by direct cytotoxic activity against cancer cells, or by modulating the tumour development process (Kintzios, 2003).

## 1.2 Traditional Medicine

### 1.2.1 Plants in medicine

Of all the plants found to have medicinal uses, none are more welcomed than those that aid in our fight against cancer. Thousands of lives have been saved or extended, by the alkaloids, vincristine and vinblastine of the Madagascaran annual periwinkle. The success of using these compounds in combination chemotherapy for treating Hodgkin's disease (80% remission), acute lymphocytic leukaemia (99% remission), Wilms' tumour (80% cured), Burkitt's lymphoma (50% cured), and gestational choriocarcinoma (70% cured) are

testimonials to the monumental strides achieved in the past, when plant products have been introduced against the most dreaded of all diseases (Lewis & Elvin, 1977). Further experimentation has indicated that mixtures of the alkaloids with other chemicals may produce even better results (Brintnall, 1986).

Over 50% of conventional drugs in clinical use are of natural products, many of which can control cancer cells. According to the WHO, more than 80% of people in developing countries depend on traditional medicine for their primary health needs. A recent survey shows that over 60% of cancer patients use vitamins or herbs as therapy. Continuous usage of herbal medicine by majority of the population in the developing countries is mainly due to the high cost of western pharmaceuticals and healthcare. In addition, herbal medicines are more acceptable in these countries from their cultural and spiritual points of view. (Kaur *et al,* 2011).

### 1.2.2 Anti-tumour agents isolated from plants

Cancer is one of the most feared diseases of our time. Pharmaceutical companies continually search for anti-cancer agents, and people afflicted with cancer, grasp at any possible panacea. The continued public pressure to allow the use of laetril (extracted from the seeds of apricot) as a treatment, despite constant documentation that it is ineffective in controlling cancer, indicates the desperation with which cures are sought. The search for truly effective anti-cancer agents has not yet been achieved. In the past few decades, a number of naturally occurring plant alkaloids were found to arrest cancer cells growth (Brintnall, 1986).

Because many alkaloids have psychoactive properties, and some are valuable medicines, they have received much attention since the 1960s. More than 5000 have been named, and alkaloids usually exist in plants as bitter-tasting, soluble, organic acid -alkaloid salts. They themselves are soluble in organic solvents, but not in water. In general, alkaloid content is

not subjected to ecological variation, and they are usually distributed throughout the plant, making all parts toxic (Saigo & Barbara, 1983).

The common periwinkle (*Catharanthusroseus*, Apocynaceae) has been used in its native range in Europe, for hundreds of years, as a folk treatment for diabetes. The plant was tested for effectiveness against various illnesses, because of its use in folk medicine. It was tried for the treatment of leukaemia in 1957, and found to be effective in curing some forms of the disease (especially those that commonly afflict children). The active chemicals, identified as vinblastine and leurocristine (vincristine) were marketed shortly after 1957, under several trade names.

Another plant found to contain anti-tumour alkaloids, is the mayapple (*Podophyllumpeltatum*, Berberidaceae). Plants of this species are herbaceous perennials that flower early in spring, in the deciduous forests of Canada and eastern U.S. The active compounds podophyllin, alpha and beta peltatin, are especially abundant in the rhizomes. Extracts of the roots were used by native Indians as purgatives and for skin disorders and tumorous growths. Now, mayapple alkaloids are used as the basis of VM-26, a drug used to treat lymphocytic leukaemia. VM-26 appears to be as effective as vincristine, in treating childhood leukaemias, although newer than the drugs based on *Catharanthus* alkaloids (Brintnall, 1986).

Finally, the plant autumn crocus (*Colchicum autumnale*, Liliaceae) has been used with some success in the treatment of cancer. The alkaloid colchicine, extracted from the corns of this species, interferes with mitotic cell division. Colchicine inhibits mitosis by interfering with development of the mitotic spindle, preventing separation of daughter chromosomes (Saigo & Barbara, 1983). As a chemotherapeutic agent, it makes use of this action by, hopefully, preventing the growth of cancer cells. However, since colchicine also affects normal cells, its use must be closely monitored, and it has produced only limited successful results (Brintnall, 1986).

Some anti-tumour agents isolated from plants, used for treating cancer traditionally, are shown in Table 1.1 below (Sofowora, 1982; Kaur *et al*, 2011).

**Table 1.1**: Anti-tumour agents isolated from plants, used for treating cancer traditionally.

| **Sr. No.** | **Plant name /Family** | **Country where used** | **Active principle** | **Class** |
|---|---|---|---|---|
| 1. | *Agapanthus africanus* Agapanthaceae | S. Africa | Isoliquiritigenin | Chalcone |
| 2 | *Aglailasylvestre* Meliaceae | India | Silvesterol | --- |
| 3 | *Ailanthus Altissima* Simaraubaceae | China | Ailnthone, Ailantenol | Quassinoids |
| 4 | *Apiumgraveolens* Umbelliferae | N. America | Apigenin | Flavonoid |
| 5 | *Bleckeriavitensis* Apocynaceae | France | Ellipticine | Alkaloid |
| 6 | *Bruceaantidysenterica* Simaraubaceae | Ethiopia | Bruceantin | Quassinoids |
| 7 | *Burseramicrophylla* Burseraceae | Mexico | Burseran | Lignan |
| 8 | *Campotheca acuminate* Nyssaceae | China | Campothecin | Alkaloid |
| 9 | *Catharanthusroseus L.* Apocynaceae | India, Africa | Vincristine, vinblastine, vindesine | Alkaloid |
| 10 | *Centaureamontata* Asteraceae | Europe | Montamine | Alkaloid |
| 11 | *Centaureaschischkinii* Asteraceae | ---------- | Schischkinnin | Alkaloid |
| 12 | *Cephalotaxusharringtonia* Cephalotaxaceae | Japan | Homo-harringtonine | Alkaloid |

| **Table 1.1**: Continued | | | | |
|---|---|---|---|---|
| **Sr. No.** | **Plant name /Family** | **Country where used** | **Active principle** | **Class** |
| 13 | *Cephalotaxusfortune* Cephalotaxaceae | China | Harringtonine, homo-harringtonine | Alkaloid |
| 14 | *Cleistanthuscollinus* Euphorbiaceae | India | Cleistanthin, Collinusin | Lignan |
| 15 | *Combretumcaffrum* Combretaceae | S. Africa | Combrestatins | Stilbenes |
| 16 | *Croton lechleri* Euphorbiaceae | S. America | Taspine | Alkaloid |
| 17 | *Daphne mezereum* Thymelaeaceae | Asia, Europe | Mezerein | ------- |
| 18 | *Diphylleiagrayi* Berberidaceae | Japan | Diphyllin | Lignan |
| 19 | *Dysoxylumbinectariferum* Meliaceae | India | Rohitukine | Alkaloid |
| 20 | *Erythroxylumpervillei* Erythroxylaceae | Madagascar | Pervilleine | Alkaloid |
| 21 | *Euphorbia semiperfoliata* Euphorbiaceae | Europe | Jatrophane | Terpenoid |
| 22 | *Fritillariathunbergii* Liliaceae | China, Japan | Zhebeinone | Alkaloid |
| 23 | *Gunneraperpensa* Gunneraceae | Brazil | 2-methyl-6(3-methyl 2-butenyl) benzo1-4-quinone | Quinone |
| 24 | *Hypericumperforatum* Clusiaceae | Europe | Hypericin | Anthra-quinone |
| 25 | *Hypoxiscolchicifolia* Hypoxidaceae | S. Africa | Hypoxoside, Rooperol | Glycoside |
| 26 | *Heliotropiumindicum L.* | India | Indicine-N-oxide | --------- |
| 27 | *Indigoferatinctoria* Leguminosae | Asia | Indirubins | Indigoids |

| **Table 1.1**: Continued | | | | |
|---|---|---|---|---|
| **Sr. No.** | **Plant name /Family** | **Country where used** | **Active principle** | **Class** |
| 28 | *Jatropha gossypiifolia L.* | Costa Rica | Jatrophone | --------- |
| 29 | *Justiciaprocumbens* Acanthaceae | India | Justicidin A,B | Lignan |
| 30 | *Lantana camara* Verbenaceae | America | Verbascoside | Glucoside |
| 31 | *Larrea tridentate* Zygophyllaceae | Mexico | Terameprocol | Lignan |
| 32 | *Linium album* Linaceae | --------- | Podophyllotoxin | Lignan |
| 33 | *Lonicera japonica* Caprifoliaceae | Japan | Luteolin | Flavanoid |
| 34 | *Maytenusbuchananii L.* | Africa | Maytansine | --------- |
| 35 | *Paris polyphilla* Trilliaceae | China | --------- | Polyphyllin |
| 36 | *Pestemondeustus* Serophulariaceae | U.S.A | Liriodendrin | Lignan |
| 37 | *Phaleriamacrocarpa* Thymelaeaceae | Indonesia | Pinoresinol, Laricinesinol | Lignan |
| 38 | *Podophyllumemodii* Berberidaceae | India | Epi-podophyllotoxin | Alkaloid |
| 39 | *Podophyllumpeltatum L.* Berberidaceae | U.S.A. | Podophyllotoxin | Glycoside |
| 40 | *Polygonumcuspidatum* Polygonaceae | Japan, China | Resveratrol | Flavanoid |
| 41 | *Pterismultifida* Pteridaceae | Japan | Pterokaurane | Terpenoid |
| 42 | *Pygeumafricanum* Rosaceae | Africa | Amygdalin | Glycoside |

| **Table 1.1**: Continued | | | | |
|---|---|---|---|---|
| **Sr. No.** | **Plant name /Family** | **Country where used** | **Active principle** | **Class** |
| 43 | *Vitexrotundifolia*, Verbenaceae | India Korea | Casticin | Flavanoid |
| 44 | *Wikstroemiaviridi* Thymelaeaceae | China | Wikstromol | Coumarin |

## 1.3 *Crinum asiaticum*

**Plate 1.1:** The plant *Crinum asiaticum* (‘tembaga suasa’ or ‘bakong’)

**Plate 1.2:** The plant *Crinum asiaticum* (‘tembaga suasa’ or ‘bakong’)

### 1.3.1 General

*Crinum asiaticum*, a large, white-flowered, lily-like plant, commonly found along sandy sea-shores, and sometimes planted inland, is occasionally cultivated for its beautiful and showy flowers. The plant is known through all Malaysia as *tembaga suasa, bakong (melong), bawang tanah, or bawang hitam.* It also grows in India, and can be found on the strand from South East Asia to western Polynesia (Quisumbing, 1951).

*C. asiaticum* has large, coated bulbs, 5 to 10 cm in diameter. The leaves are crowded at the apex, lanceolate, 90 to 150 cm long, 12 to 15 cm wide. Its scape, arising from the axils of the old leaves, is erect, stout, and solid, 1 m high, or less. The spathe subtending the flowers, is about 15 cm long. Its flowers are fragrant, 20 to 40, each subtended by a thin, narrow, bracteole. The perianth tube is greenish, and about 1 cm long; and the lobes are spreading,

white, linear, recurved or revolute, about 8 cm long, and 8 mm wide. Its filaments are very slender, free, and purplish above. The fruits are sub-globose, about 5 cm in diameter.

The substance acting emetically, was reported to be an alkaloid, lycorine (1 to 1.8 %), which is allied to emetine. The astringent property is due to the presence of a considerable quantity of tannin (Quisumbing, 1951).

### 1.3.2 Uses of *Crinum asiaticum* in traditional medicine

The parts of the plant used medicinally, are the leaves and the bulbs, which are mentioned in the Pharmacopoeia of India (Quisumbing, 1951). The bulbs are prepared as an ointment, and the leaves are used as an emollient, both in the form of topicals. The bulbs have emetic properties. The leaves and roots are also emetic and diaphoretic, and considered a good substitute for ipecacuanha. Moreover, they act without gripping, purging, or any other distressing symptoms. The succulent leaves, besmeared with castor oil, and warmed, or the bruised leaves, mixed with the oil, form a useful application for repelling witlows, and other inflammations at the ends of toes and fingers; also as formentations on inflamed joints and sprains. Another use of the leaves (juice with a little salt) is for earache, and other ear complaints. After being slightly heated, an oil is also prepared from the fresh juice, and used for the same purposes.

According to The Indian Materia Medica (Quisumbing, 1951), the roasted bulb is used as a rubefacient in rheumatism. The bulbs also have been used in India, as tonics, laxatives and expectorants, and in urinary troubles, whilst the seeds are applied for purgatives and diuretic actions. The leaves are also used as expectorants, and against skin diseases (Patel *et. al*., 2010).

*Crinum asiaticum* does not appear to be used as an internal medicine by the Malays. In Malaysian state of Pahang, the Malays oil the long smooth leaves of ‘bakong’ and, after