CHAPTER 2

LITERATURE REVIEW

2.1 Natural products

The organic compounds synthesized by plants are known as plant natural products or secondary metabolites. Plant secondary metabolites are usually classified according to their biosynthesis pathways (Harborne, 1999). The three large families of secondary metabolites include phenolics, terpenes and alkaloids contribute to plant fitness by interacting with the ecosystem. They prevent predators, destroy other plants that compete for the same space and attract pollinators (Yuan and Lin, 2000). Some of these metabolites have anti-cancer, anti-biotic, anti-fungal and anti-viral properties (Bourgaud *et al.*, 2001). Due to their inherent biological activities, plants that contain secondary metabolites have been used as medicines in some countries many thousands of years ago. Conventional cancer therapies cause serious side effects and at best merely expand the life of a patient by a few years (Amin *et al*., 2009). This has brought new interest in natural product research due to the failure of alternative drug discovery (Butler, 2004). The advantage in using natural compounds lies in their relatively non-toxic nature and defined mechanism of action (Amin *et al.*, 2009). Following the discovery of the vinca alkaloids (vinblastine and vincristine), the hunt for anticancer agents from plant natural products started in earnest in 1958 (Mishra, 2011).

2.2 Cancer

Cancer can be described as a disease in which cells grow and spread unrestrained throughout the body and eventually causing death. Cancerous cells will invade neighboring tissues and to the other parts of the body, and thus is potentially life threatening (Becker *et al*., 2003). There are more than 100 different kinds of cancers and the cells can develop in almost any organ, fluid or tissue. Certain cancers affect blood cells, skin, others affect bone, muscle or nerve tissue. The major characteristic of cancer cells is lack of control of cell proliferation, differentiation and death invading organs and tissues. Cancer can be categorized into carcinomas, sarcomas, leukemias, lymphomas and central nervous system cancers (NCI, 2009). Carcinomas are cancers that develop from the epithelial cells that form covering layers over external and internal body surfaces. They are the most common type of malignant tumor. Sarcomas are cancers that occur in slowly dividing tissues which originate in supporting tissues such as cartilage, bone, blood vessels and muscle. They are the rarest types of human cancers. Meanwhile, leukemia and lymphomas are cancers which develop from lymphatic cells and blood origin.

Cancer is mainly caused by mutations in the DNA. Some cancers are triggered by DNA damaging chemicals (benzene or nickel) and radiation (UV-radiation or X-rays), whereas other arise from spontaneous DNA mutations and replication errors. Mutation in certain genes such as proto-oncogenes like ras and tumour suppressor gene like p53 will influence the regulation of fundamental cellular processes like proliferation, differentiation and apoptosis (Scholzova *et al*., 2007; Kintzios and Barberaki, 2003). Certain mutations are inherited and expose the individual to a higher risk of cancer. Certain cancers are also related to viral infection, such as Human Papilloma Virus and Epstein-Barr virus.

Cancer development or also known as carcinogenesis is a multistep process and these steps are sign of genetic alteration that generates the progressive transformation of normal cells to highly malignant tumours. Basically, carcinogenesis can be divided into initiation, promotion and progression stages (Ito *et al*., 1995). Metabolism may inactivate or activate the carcinogen, resulting in the carcinogen interacting with DNA to produce DNA adducts and lesions. While an ineffective DNA repair mechanism may allow these mutations or adducts to propagate during cell division. Proliferation permanently embeds the change in the genome (Tannock *et al*., 2005). During promotion stage, the initiated cells show altered phenotypic expression that support rapid proliferation in a supportive environment that maintains the proliferative potential (Ito *et al*., 1995). The final stage is progression, where an array of consequences for the tumour cell ensues. Instability of genetic which occurred in this stage will lead to amplification, transclocation and rearrangements of chromosome or gene. For example, deletions may cause proto-oncogene activation and tumour suppresser gene (p53) inactivation. These alterations allow the cancer cells to acquire malignancy and survive in the human body (Ruddon, 2007).

Cancer cells generate six alterations in cell physiology to defect the regulatory circuits that govern normal cell proliferation and homeostasis. Besides that, these alterations let the cancer cells obtain novel capabilities to successfully circumvent the anticancer defense mechanisms of the body. These alterations are self-sufficient growth signals, insensitivity to antigrowth signals, evasion of apoptosis, endless replication, sustained angiogenesis, tissue invasion and lastly metastasis which lead to death (Hanahan and Weinberg, 2000).

2.3 Apoptosis in cancer

Apoptosis known as programmed cell death or cell suicide programme, can be characterized as energy dependent biochemical mechanism. Apoptosis is an essential requirement for embryogenesis, organ metamorphosis, deletion of damaged cells and tissue homeostasis. Cells defective in apoptosis are the main cause of complex diseases, for example cancer, neurodegenerative disease and autoimmune disorder (Fadeel *et al*., 1999). In general, apoptosis can be characterized by morphological and biochemical changes. The morphological changes include cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and apoptotic bodies formation (Reed and Tomaselli, 2000) whereas the biochemical changes are discrete DNA fragmentation, activation of caspases and the presentation of phosphatidyserine on the outer surface of the cell membrane (Tan *et al*., 2009).

Apoptosis is different from what happens when cells are destroyed by physical injury or exposed to certain poisons. In response to such non-specific damage, cells endure necrosis, a slow type of death in which cells swell and eventually burst, spewing their contents into surrounding tissues. Necrosis can result in a local inflammatory reaction that can cause further cell damage, which makes it potentially dangerous. Apoptosis, on the other hand kill cells quickly and neatly, without causing damage to the nearby tissue (Kleinsmith, 2006). Many researches recognized induction of apoptosis as one of the efficient strategies in cancer chemotherapy and a very important property of a candidate anti cancer drug (Taraphdar *et al*., 2001).

The mechanism of apoptosis is divided into two pathways known as the intrinsic pathway (mitochondria pathway) and extrinsic pathway (death receptor pathway) (Elmore, 2007). Extrinsic pathway is employed when a cell has been targeted for destruction by other cells in the surrounding tissue. In such cases, molecules that

transmit a ‘death signal’ were produced by the neighboring cells by binding to death receptors present on the outer surface of the targeted cell. The activated death receptors then interact with, and trigger activation of, initiator procaspase molecules located inside the cell, thereby starting the caspase cascade (Kleinsmith, 2006).

Meanwhile, intrinsic pathway is a pathway that is mainly relevant to the cancer biology field, where it functions mainly in the destruction of cells that have sustained extensive DNA damage. Apoptosis is induced in cells that have sustained extensive DNA damage and the p53 protein plays a critical role. The presence of damaged DNA triggers the accumulation of the p53 protein, which helps in the production of proteins that alter the permeability of mitochondrial membranes. This leads to the release of a group of proteins, particularly cytochrome c, which activates the caspase cascade and eventually destroy the cells by apoptosis (Kleinsmith, 2006).

2.4 Chemoprevention of cancer

According to Sporn (1976), cancer chemoprevention was defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression. The other definition for cancer chemoprevention is the use of agents to slow the progression of, reverse, or inhibit carcinogenesis, thus lowering the risk of developing invasive or clinically significant disease (Hong and Sporn, 1997; Kelloff *et al*., 2001). Chemopreventive agents can be divided into three categories. The first category consists of compounds that prevent the formation of carcinogens from precursor substances. The second categories are the compounds that inhibit carcinogenesis by preventing carcinogenic compounds from reaching or reacting with the critical sites in the tissues. Lastly the third category consists of inhibitors which act subsequent to exposures to carcinogenic agents. These inhibitors are termed

"suppressing agents" (Wattenberg, 1985). An effective chemo preventive agent should intervene early in the process of carcinogenesis to eliminate premalignant cells before they become malignant (Smith *et al*., 1995; Kelloff *et al*., 1999).

Criteria that an ideal chemopreventive agent should have are significant reduction in cancer development, no or minimal side-effects, ease of administration, known mechanism of action and cheaper cost. There are two strategies for the development of chemopreventive agent. The first one is to identify the natural diet-derived agents in cancer incidence and mortality, geographic variations and migration related to changes in dietary and lifestyle practices. The second one is to design and synthesize molecular target-based agents. These require isolation, characterization and preclinical evaluation of test agents for their development as chemopreventive agent (Gupta, 2007).

2.5 The Zingiberaceae family

Zingiberaceae is one of the largest families in the order Zingiberales which comprises about 1200 species. Among the plant families, the family Zingiberaceae is wildly distributed throughout the tropics especially in Southeast Asia and a component of herbaceous ground flora of the rainforest in Peninsular Malaysia. There are about 150 species of ginger belonging to 23 genera found in Peninsular Malaysia (Holttum, 1950). Zingiberaceae species usually grow naturally in damp, shaded parts of the lowland or on hill slopes, as scattered plants or thickets. It is easy to recognize most members from this family by the characteristic aromatic leaves and fleshly rhizome when they are crushed and also by the elliptic to elliptic-oblong leaves arranged in two ranks on the leaf-shoot (Habsah *et al*., 2000; Hasnah, 1991). The flowers are delicate, empimeral and highly modified (Sirirugsa, 1997) with unique aesthetics which serves as a commodity for ornamental plants.

There are few species of Zingiberaceae which are used as spices, medicines, flavouring agents and as the source of certain dyes in the Southeast Asian region (Burkill, 1966). A variety of Zingiber species are also popularly consumed fresh by village folk as a local salad known as *ulam.* In respect to its medical application, Zingiberaceae have been used in traditional preparation such as *jamu* for the treatment of common ailments (Halijah and Ahmad, 1988; Hasnah, 1991). Some species from the genera *Alpinia, Amomum, Curcuma, Costus, Kaempferia* and *Zingiber* are main ingredients in traditionally prepared tonics locally known as 'Jamu', which are commercially available. Various Zingiberaceae plants have been used traditionally to treat conditions associated with women-related illnesses, flatulence, gastrointestinal disorders, stomach ache and nausea among others (Larsen *et al.*, 1999; Kikuzaki, 2000).

2.6 The *Alpinia* genus

The genus *Alpinia* was first classified by Plumier, but it was named after the 16th century Italian botanist Prospero Alpino. This genus belongs to the division Magnoliophyta (Angiospermae), the subclass Zingiberidae and the order Zingiberales. *Alpinia* is one of the largest and taxonomically complex genus from the Zingiberaceae family with 230 species recorded throughout tropical and subtropical Asia (Kress *et al*., 2005).

According to Ridley (1924), twenty three species of *Alpinia* were found in Malay Peninsula. *Alpinia* species is medium to large sized forest plants with some species reaching a height of over three meter and it is the only genus that has a terminal inflorescence on the leafy shoots (Larsen *et al.*, 1999). According to Almeida (1993), the beauty of its inflorescence is the most remarkable trait of this genus and explains its wide ornamental use through the commercialization of its seedlings and flowers.

Some studies showed that the leaves of some *Alpinia* species perform as plant growth inhibitors and have insecticidal and antifungal activities (Tawata *et al.,* 1996). Besides that, their rhizomes also have inhibitory activity against the actions of histamine and barium chloride on excised guines pig ileum (Hsu *et al.,* 1994). Whereas, the seeds from some of these plants can be used as aromatic stomachic, carminative, astringent, tonic and sedative drugs (Itokawa *et al.,* 1980). Many *Alpinia* plants are medicinal herbs and studies have shown that they possess antioxidant, anti-inflammatory, anticancer, immunostimulating, hepatoprotective and antinociceptive activities (Chang *et al*., 2010).

Sirirugsa (1999) describes that *A. conchigera* used for the treatment of bronchitis, rheumatism and arthritis, while Ibrahim *et al*. (2009) detected a broad spectrum of antibacterial and antifungal actions in that species. In some African tribes, the species *A. smithiae* is used in the therapeutic treatment of both humans and cattle (Joseph *et al*., 2001). Besides using in traditional medicine, some of the *Alpinia* species were used in the preparations and flavorings of food in many Asian countries. For example, the rhizomes of *A. galangal* are used as spice or substitutes for food flavoring and stomachache treatment in Thailand and China (Matsuda *et al*., 2003) and coughs, asthmas, bronchitis, headache, inflammation, rheumatoid arthritis and colic in Malaysia (Burkill, 1966). In Okinawa, Japan, one of the staples of the local diet is made from rice and *A. zerumbet* leaves and the rhizomes are used to make seasonings and beverages.

2.6.1 Phytochemical and bioactivity of *Alpinia* species

Some phytochemical work and biological study have been reported on *Alpinia* species. *Alpinia* species were reported to contain bioactive components such as chalcones and flavonoids (Giang *et al*., 2005), sesquiterpenes (Miyazawa *et al*., 2000), labdane diterpenes (Sy and Brown, 1997), kava pyrone (Mpalantinos*et al*., 1998) and diarylheptanoids (Miyazawa *et al*., 2000). Diarylheptanoids are present in several

Alpinia species and related to anti-inflammatory activities by interfering with the biosynthesis of prostaglandins and leukotrienes, as well as interfering with the production of nitric oxide in macrophages (Yadav *et al*., 2003). The leaves and roots of *A. zerumbet* contain kavain and dehydro-kavain (Kuster *et al.,* 1999). Some flavonoids such as cardamomin and alpinetin have already been identified in rhizomes and seeds of the species *A. zerumbet* and *A. katsumadai* (He *et al.*, 2005).

Besides that, the genus *Alpinia* has been widely studied for its cancer-fighting properties. Different substances from these species have been reported as having a beneficial effect in preventing and treating cancer (Surh, 1999). For instance, the essential oil from the species *A. oxyphylla* has proven effective against cancerous lineages (Lee and Houghton, 2005). Furthermore, Hahm *et al*. (2003) reported the action of substances extracted from the seeds of *A. katsumadai*, which were cytotoxic for cell lineages of human lung cancer and leukemic lineages. Rhizome extracts from *A. officinaram* were found to be efficient in inhibiting melanogenesis in studies with B16 melanoma cells (Matsuda *et al*., 2009). Extracts of *Alpinia* species have been applied in the treatment of inflammatory processes. The species *A. officinarum* was proved to be effective in the therapeutic or preventive treatment of acute or chronic arthritis (Lee *et al*., 2009) while blepharocalyxins C-E isolated from *A. blepharocalyx* was reported to have cytotoxic activity against human HT 1080 fibrosarcoma and highly liver-metastatic murine colon 26-L5 carcinoma cells (Tezuka *et al*., 2000). The dichloromethane extract of *A. officinarum* and *A. galangal* showed strong toxicity towards the human non-small cell lung cancer (COR L23) and the human adenocarcinoma (MCF7). Furthermore, 1'- acetoxychavicol acetate which was isolated from both plants was the major cytotoxic component against COR L23 and MCF7 (Lee and Houghton, 2005). According to Banjerdpongchai *et al*. (2011), 4'-hydroxycinnamaldehyde (4'- HCA) which was isolated from *A. galangal* was cytotoxic

to human leukemic HL60 and U937 cell lines in a dose-dependent manner. Besides that, *A. galangal* extract at a dose of 300 µg/ml caused apoptosis of all cell lines including normal and p53-inactivate fibroblasts, normal epithelial and tumour mammary cells and a lung adenocarcinoma cell line (Muangnoi *et al*., 2007).

2.7 The *A. scabra* (Blume) Náves

A. scabra can reach up to 2 to 3 m tall when flowering. The leaves are 40-50 cm, oblong, edges with scattered stiff hairs, apex rather shortly acuminate, base cuneate, lower surface short hairy or sometimes almost glabrous. Petiole to about 1 cm long, ligule to 1 cm long, short hairy or glabrescent. The inflourescence are 30 to 40 cm long or more long, usually with 2 or 3 large branches (to 15 cm long) in the lower part, the branches in the axils of long sheaths; aptical portion, bearing short cincinni only about 20 to 30 cm long; rachis rather stout, short hairy or almost glabrous. Primary bracts towards base of inflorescence are very small, towards apex up to 8 mm long. Stalks of cincinni are 1 to 2.5 cm long; up to 6 flowers on each. Secondary bracts about 1 mm long and pedicel slender about 5 mm long. Ovary at flowering about 1 mm long and calyx 5 mm long, broadly, tubular, white, unequally 3-lobed, tips of lobes shortly pointed, hairy. Corolla tube slender, 8 mm and lobes about 10 mm long and white. Labellum shorter than the corolla lobes, white, cleft almost to the base, the two halves bilobed with narrow apical with wider lateral lobe. Filament elongating to nearly 1 cm; another 5 mm long with a small crest. Staminodes hardly 1 mm long, tooth like, at base of tip and the fruit is round, smooth, black, 10 to 12 mm diameter, containing few seeds (Holttum, 1950).

In Perak, a hot water fomentation is made with *A. scabra*, or heated leaves are applied to the abdomen to treat vertigo (Burkill, 1935). There is little information available in the literature about *A. scabra* and the first report on the cytotoxic effect of

A. scabra was by Ibrahim *et al.* (2010). Based on the preliminary data obtained, *A. scabra* is considered as an agent with potential anticancer activity. The appearance of *A. scabra* is shown in Figures 2.1, 2.2, 2.3 and 2.4.

Figure 2.1: The appearance of *A. scabra* in Genting Highlands, Pahang, Malaysia

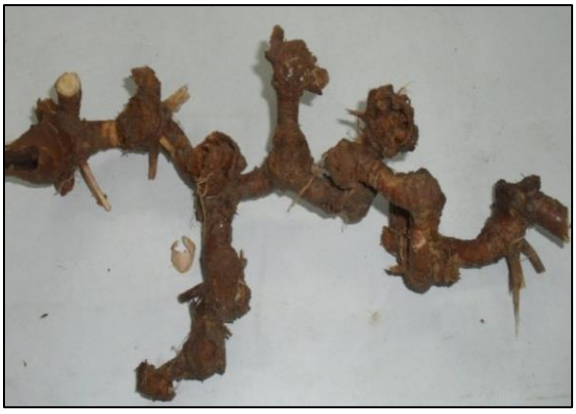

Figure 2.2: The rhizomes of *A. scabra*

Figure 2.3: The roots of *A. scabra*

Figure 2.4: The pseudo stems of *A. scabra*

2.8 Bioassay-guided isolation

Bioassay-guided isolation has been used as a rapid and successful method to isolate pure bioactive compounds from natural products, either from plants, microorganisms or fungi in the process of drug discovery. This method uses bioassay to monitor the activity of fractions generated from chromatography separation steps results in the isolation of bioactive compounds (Rimando *et al.*, 2001). In this endeavour, the crude extracts of natural products are separated into many fractions in the process of bioassay-guided isolation. After that, bioassay of each fraction is performed to determine which of them should be subjected to further separation. Through the circle of separation and bioassay, a pure active compound may be isolated (Cheng *et al.*, 2006).

The bioassay can be described as the biological system to detect properties of the crude extract, chromatographic fraction, mixture or pure compounds. Bioassays include anti-bacterial, anti-HIV, anti-fungal and anti-cancer assays (Sarker *et al.*, 2006a). The anticancer properties of the extracts can be identified using cytotoxic assays which measure the capability of drugs to kill cells. These assays are based on the parameters, such as metabolic activity and cell morphology. For example, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay to measure mitochondrial activity, lactate dehydrogenase leakage assay to investigate membrane integrity, neutral (3-amino-m-dimethylamino-2-methylphenazine hydrochloride) red assay to measure the viable cell after exposure to toxicant, ATP measurement and macromolecular synthesis and glutathione depletion (Weyermann *et al.*, 2005). Bioassay can be done by using either *in vitro* (cell culture) systems, *in vivo* (clinical trial, whole animal experiment) or *ex in vivo* (isolated organ or tissue). *In vivo* studies are more related to clinical applicability and also provide the toxicity data of the compound against humans or animals. However *in vitro* bioassays can quickly identify

the bioactivities of the extract or compounds in a short period of time (Sarker *et al*., 2006b).

The purification or separation of compounds can be done using chromatography techniques, such as gravity column chromatography, thin layer chromatography, and high performance liquid chromatography (HPLC). The purified compound can be indentified using spectroscopic methods like fourier transform infrared spectroscopy, mass spectrometry and nuclear magnetic resonance spectrometry (Pieters and Vlietinck, 2005).

2.9 *In vitro* cytotoxic activity assays

Cytotoxicity assays are widely used in *in vitro* toxicology studies. *In vitro* cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals (Scheers *et al*., 2001). According to Wilson (2000), the application of *in vitro* assays systems for the search of potential anticancer agent is common for the beginnings of cancer chemotherapy since 1946. The use of cell culture systems basically offers numerous advantages over the whole animal model (*in vivo*), being inexpensive, sensitive, rapid, reproducible of the test conditions, no ethical constraint associated with using animal experimentation and could further curtail the use of animals for LD_{50} tests (Fotakis and Timbrell 2006).

In past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). The most convenient, modern assays have been optimized for the use of microtiterplates (96-well format) and this allows many samples to be analyzed rapidly and simultaneously. Colorimetric and luminescence based assays allow samples to be measured directly in the plate by using a microtiterplate reader or ELISA plate reader. Cytototoxcity assays have been developed which use different parameters associated with cell death and proliferation.

Previous reports have shown that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (Wayermann *et al*., 2005). The lactate dehydrogenase leakage (LDH), neutral red, the methyl tetrazolium (MTT) or Sulforhodamine B (SRB) assays are the most common employed assays for the cell viability following exposure to toxic substances (Fotakis and Timbrell 2006). Thus, it is important to consider what effect is expected, respective of what cell death mechanism is predicted and one has to be careful with the suitability of the assay used to prevent false-positive or false-negative results (Wayermann *et al*., 2005). In this study, MTT assay according to the method described by Mosmann (1983) was utilized to determine the cytotoxic activity.

2.10 Apoptosis screening and detection

There are some methods that can be used for apoptosis screening and detection. In this study, detection of morphological changes was done using phase-contrast inverted microscope and DAPI nuclear stain whereas DNA fragmentation was done using agarose electrophoresis.

2.10.1 Detection of morphological changes

Microscope techniques is one of the methods used to detect apoptosis by observing the morphological changes. These includes electron microscopy, light microscopy and fluorescence microscopy. Electron microscopy is time consuming and requires expensive equipment and specialized training (White and Cinti, 2004) meanwhile light and fluorescence microscope can be used to detect the late events of apoptosis (Huerta *et al.*, 2007). These microscopic techniques can detect apoptosis *in vitro* but they can only detect apoptosis at a single point in time and inability to screen a

large number of samples. So, it is possible to miss the characteristic apoptosis bodies by microscopy to detect apoptosis.

A large number of fluorescent dyes can be used to stain live or dead cells and these dyes can be used for cell counts under epifluorescence illumination and/or they can be used for analysis using a fluorescence plate reader. Examples of fluorescent DNA binding dyes are DAPI, propidium iodide, acridine orange, chromomycin, and others and these dyes may be used in conjunction with light counterstain, or alternate sections may be stained with another dye, antibody, or reagent of interest. When examined using the appropriate excitation lamps and detection filters, specific staining of the nuclei allows clear visualization of the difference between a viable cell with a normal sized, diffusely stained nucleus and an apoptotic cell, with smaller solid spheres or blobs or condensed chromatin. Most fluorescent DNA binding dyes do not penetrate the outer membrane of living cell and thus can be used only on fixed specimens. Due to apoptotic cells maintain membrane integrity during at least the initial stages of death, they are not permeable to most stains prior to fixation (Electra *et al.*, 1995).

2.10.2 Detection of DNA fragmentation

Formation of DNA fragmentation is one of the characteristic features observed in apoptotic cells and it is generally considered as the biochemical hallmark of apoptosis (Zhang and Xu, 2002). The formation of a DNA ladder correlates with the early morphological signs of apoptosis and has been widely used as a distinctive marker of the apoptosis process (Wylie *et al.,* 1984). Fragmentation of DNA progress from high molecular weight (300-700 kbp) fragments to fragments of size around 50 kbp (Zhivotovsky *et al.*, 1994). At late stages of apoptosis fragmentation of DNA in intranucleosomal regions may also occur, giving rise to a characteristic “ladder” of fragments as revealed by electrophoresis in agarose gels (Wyllie, 1980).