POTENTIAL OF *Stichopus horrens* AND *Stichopus vastus* EXTRACTS AS ANTICANCER AND WOUND HEALING AGENTS: EFFECTS ON BREAST CANCER AND FIBROBLAST CELL LINES

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By

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In the name of Allah,

The most beneficent the most merciful.

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ASR	Age standardized incidence rate
ATCC	American Type Culture Collection
BAK	BCL-2 antagonist
BAX	BCL-2 associated X protein
BCRA2	Breast cancer type 2 susceptibility protein
BHT	Butylated hydroxytoluene
BRCA1	Breast cancer type 1 susceptibility protein
C33A	Human cervical cancer
Caco-2	Human epithelial colorectal adenocarcinoma
$CHCl_3$	Chloroform
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2 –diphenyl -1-picrylhydrazil
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FADD	Fas-associated death domain

FAS	Type-II transmembrane protein
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GAGs	Sulphated Glycosaminoglycans
HFF-1	Human normal foreskin
MCF 10A	Non-tumorigenic breast epithelial
MCF 7	Estrogen positive breast adenocarcinoma
MDA MB231	Tumorigenic breast adenocarcinoma
MeOH	Methanol
MMPs	Matrix Metalloproteinase
NCCD	Nomenclature Committee on Cell Death
NCR	National Cancer Institute
NIH/3T3	Swiss mouse embryo fibroblast
OPTIMEM	Reduced-Serum Medium
PBS	Phosphate Buffer saline
PI	Propodium iodide
PS	Phosphatidyl serine
PUFA	Polyunsaturated fatty acids
ROS	Reactive Oxygen Species

POTENSI EKSTRAK *Stichopus horrens* DAN *Stichopus vastus* SEBAGAI AGEN ANTIKANSER DAN PENYEMBUHAN LUKA: KESAN KE ATAS TITISAN SEL KANSER PAYUDARA DAN FIBROBLAST

ABSTRAK

Eksploitasi komersial ke atas timun laut disebabkan oleh peningkatan amalan gaya hidup sihat di kalangan masyarakat telah banyak mendapat pengiktirafan di kalangan pengguna, industri farmaseutikal dan para penyelidik. Di semenanjung Malaysia, kegunaan timun laut tempatan yang juga dikenali sebagai gamat (*Stichopodidae*) dalam perubatan telah lama dieksplotasi. Walau bagaimanapun, pengetahuan dari sudut saintifik berkenaan peranan *Stichopus horrens* dan *Stichopus vastus* dalam bidang perubatan dan sama ada kedua-dua spesis ini memiliki unsur bioaktif yang berpotensi amatlah sedikit. Dalam kajian ini, *Stichopus horrens* dan *Stichopus vastus* telah diekstrak menggunakan pelarut buffer fosfat dan klorofom : metanol. Keupayaan ekstrak untuk memberi kesan rencatan terhadap sel kanser payudara MCF-7 dan MDA MB 231 telah dinilai melalui ujian MTS. Ekstrak *Stichopus vastus* buffer fosfat didapati paling sitotoksik keatas sel kanser payudara, MCF-7 dengan nilai IC_{50} (65.14±5.59µg/ml) berbanding kesan keatas sel kanser payudara MDA MB231 (73.09±7.32µg/ml). Kami juga mendapati ekstrak *Stichopus vastus* buffer fosfat adalah 'cytoselective' terhadap sel kanser dan tidak menghalang pertumbuhan sel payudara normal (MCF-10A) pada nilai kurang dari 200µg/ml. *Stichopus vastus* buffer fosfat ekstrak didapati menyebabkan kematian sel secara apoptosis melalui pengikatan 'phosphatidylserine'

pada bahagian luar sel yang mangalami kojugasi. Walau bagaimanapun, *Stichopus horrens* tidak menunjukkan kesan sitotoksik ke atas sel kanser payudara, MCF-7 dan MDA MB231 oleh itu, potensi bioaktif spesis ini dinilai dari sudut berbeza. *Stichopus horrens* buffer fosfat ekstrak dan metanol ekstrak didapati berpotensi dalam mempercepatkan proses penyembuhan luka pada kepekatan tertentu. *Stichopus horrens* metanol ekstrak juga juga didapati membantu dalam mengurangkan penularan sel kenser payudara MDA MB231, ke kawasan lain pada kepekatan 50 μg/ml. Berdasarkan kajian kami, *Stichopus vastus* mempunyai potensi dalam bidang anti kanser dan sesuai digunakan sebagai produk aktif farmakologi dan agen kemoterapeutik. Manakala *Stichopus horrens* mungkin berpotensi dalam memainkan peranan sebagai pencegah penularan sel kanser payudara dan mempercepatkan penyembuhan luka.

POTENTIAL OF *Stichopus horrens* AND *Stichopus vastus* EXTRACTS AS ANTICANCER AND WOUND HEALING AGENTS: EFFECTS ON BREAST CANCER AND FIBROBLAST CELL LINES

ABSTRACT

The commercial exploitation of sea cucumbers biomass due to healthy lifestyle are gaining much recognition among consumers, pharmaceutical industries and researchers. In peninsular Malaysia, the medicinal uses of sea cucumbers locally known as 'gamat' (Sea cucumbers of the *Stichopodidae* family), have been exploited. However there is still lacking of scientific research published regarding *Stichopus horrens* and *Stichopus vastus* biomass role especially for medicinal porposes, and whether this invertebrate actually possess bioactive compounds. In this study, *Stichopus horrens* and *Stichopus vastus* have been extracted using phosphate buffer saline and chloroform: methanol solvent. The ability of different crude extract obtained to induce cell death on MCF-7 and MDA-MB-231 cell lines was determined using MTS assay. *Stichopus vastus* PBS extract was found to be most cytotoxic on MCF-7 cells with IC_{50} value (65.14±5.59µg/ml) compare to MDA MB231 (73.09±7.32µg/ml). We also found that the *Stichopus vastus* PBS extract is cytoselective towards cancerous cells and does not inhibit the proliferation on MCF 10A at concentration less than 200µg/ml. The *Stichopus vastus PBS* extract induced the cell death via apoptosis was demonstrated by binding of phosphatidylserine on the conjugated cell membrane. However for *Stichopus horrens* extracts, the analysis was focused on others bioactive potential because the MTS assay

results did not show potential effect on inhibit the proliferation of the cancer cells, MCF-7 and MDA MB 231 cells. The *Stichopus horrens* PBS and methanol extract were found to have good potential in wound healing process by accelerating the wound closure at certain concentration. *Stichopus horrens* methanol extract also helps to reduce the invasion of MDA MB 231 cells at concentration 50ug/ml. Based on our study, *Stichopus vastus* has potent anticancer activities and could therefore, potentially be a source for a pharmacogical active product suitable for development as chemotherapeutic agent and *S. horrens* may be able to play a significant role in prevent the invasion of the breast cancer cells and induce the wound healing process.

CHAPTER ONE

INTRODUCTION

1.1 Stichopus *sp*.

1.1.1 Taxonomy and Morphology

Kingdom	:	Animalia
Phylum	:	Echinodermata
Class	:	Holothuroidea
Family	:	Stichopodidae
Genus	:	Stichopus
Species	:	*Stichopus horrens* (Selenka, 1867) *Stichopus vastus* (Sluiter, 1887)

Holothuroids or also known as sea cucumbers are abundant and diverse group of marine invertebrates (Ridzwan, 2007). The sea cucumbers were known as soft bodied creatures and characterized with flexible, worm like, gelatinous body and similar morphology as a cucumber (Broadbar *et al*., 2011; Moriarty, 1982). Their endoskeleton is much more reduced that most echinoderms and they lack the spines compare to other echinoderms. Sea cucumbers belonging to the family of Stichopodidae have either a square shaped or trapezoidal cross section and their ossicles are in the form of C-, S-, and branched shaped rods. In the genus of *Stichopus*, the bottom is slightly flattened and is usually covered with podia or tube feet. The body is also covered by tuberculues and papillae (Moriarty, 1982). The morphological characteristic of the sea cucumber is the main method for species identification and among the characteristic that need to be

emphasized are body shape, body colour, the existence and shape of papillae on both dorsal and ventral parts of sea cucumbers (Kamarudin *et al*., 2009).

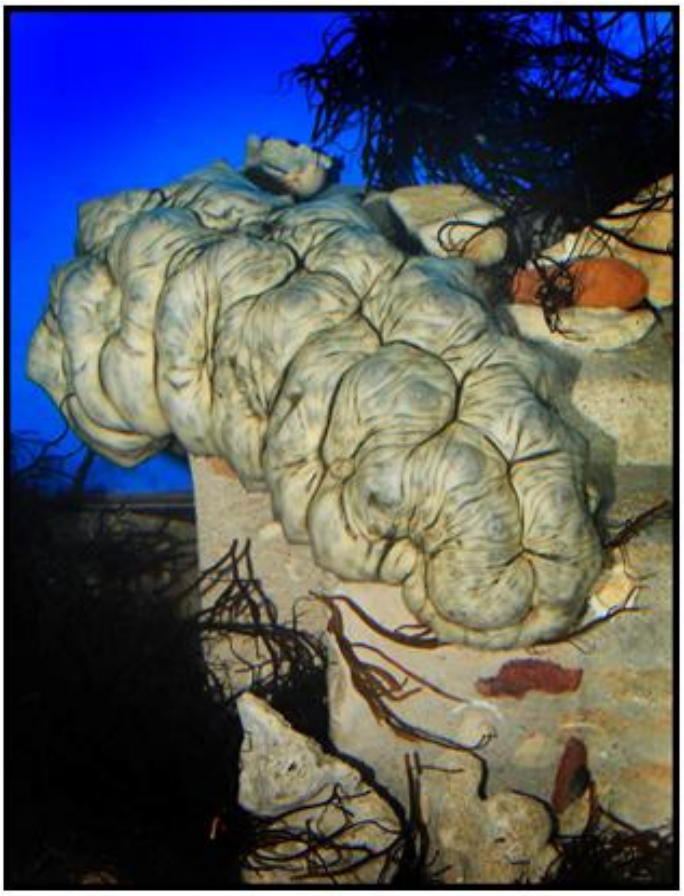

Figure 1.1: A digital image of the *Stichopus vastus* species [electronic print] Available at: http://www.gaiaguide.info/lirs/Group.html?groupId=G9nJaURt [Accessed 20 March 2013].

Figure 1.2: A digital image of the *Stichopus horrens* species [electronic print] Available at:http://www.scuba-equipment-usa.com/marine/AUG06/Stichopus_spB .html [Accessed 20 March 2013].

Kamaruddin (2009) in his publication has stated that there are four species of sea cucumber in Malaysia that commonly called as '*gamat', Stichopus chloronatus, Stichopus horrens (gamat emas), Stichopus ocellatus,* and *Stichopus vastus*.

1.1.2 Distribution and exploitation in Malaysia

Sea cucumbers are slow moving animals, living on the base substrate of sand, near corals, rocks or sea weeds in warm shallow waters (Conand, 1990). Recent studies on distribution of sea cucumber in Malaysia recorded that around 8% from 50 species of sea cucumber found in Malaysia is from genus *Stichopus*. Others genus including *Holothuria*, *Actinopya, Bohadschia*, *Molpadia* and *Pearsonuthuria* (Kamarudin *et al*., 2009). This finding is supported by Baine who identified five genera of order *Aspirochorotida* namely *Holothuria, Stichopus, Thelenota, Bohadschia* and *Actinopyga* are the most abundant and top species diversity within Malaysia coral reef habitats (Baine and Forbes, 1998). Low diversity of sea cucumber has been observed along the west coast of peninsular Malaysia this may the sequences of the less distribution of coral reef and the poor underwater visibility (Baine and Sze, 1998). In Pulau Langkawi on the west coast of Peninsular Malaysia, *Stichopus horrens* has been one of the most endangered species because of the high demand in processing industry on producing medicinal products (Choo, 2004). There are variety of sea cucumbers based products offered in Langkawi such as lotions, water, toothpaste, oils, tablets and cosmetics (Wen *et al*., 2010; Yaacob *et al*., 1997). Sea cucumber become so popular in Langkawi, when farmers from Langkawi visited the island of Adang in Thailand and being surprised with the healthy look of the local peoples. They then being told about the valuable properties of sea cucumber and start to practice and expand the usage of sea cucumber. In 1940s,

there was a higher demand and Langkawi start to import sea cucumber from Adang fisherman in Thailand. Until now, Langkawi still imports around 30 boats of dried gamat every season (October to February) with every boat contain 300kg of dried gamat. Other area with well-known fishery of sea cucumber is Sabah where they also export sea cucumber to overseas market as China, Taiwan, Singapore, Hong Kong, Japan, Korea and Thailand (Baine and Forbes, 1998). Sea cucumber fishery in Asia is one of the most demanding field that contributing to improve economic situation, therefore to achieved the stability and sustainable source, the population of sea cucumber should be managed appropriately (Conand, 2008; Choo, 2008; Tuwo, 2004). It has been estimated that harvestable catches for Asia and Pacific region are in the range of 20, 000 to 40, 000 tons per year.

1.1.3 Biological studies and therapeutic value on sea cucumber

Sea cucumbers are one of the potential marine organisms with high therapeutics value. The medicinal properties of this animal are recognized by presence of various beneficial components with promising biological activities. Located in the same genus *Stichopus* , a lot of isolation and purification of potential bioactive components has been done on this group. However there is still a lack of scientific studies on the amount of bioactive components in *Stichopus horrens* and *Stichopus vastus.*

Sulphated Glycosaminoglycans (GAGs) has been claimed by Chen (2003), as a reason behind these valuable medicinal values of sea cucumber. Glycosaminoglycans or also known as mucopolysacharide are huge complex carbohydrate molecules that interact with a wide range of proteins involved in physiological and pathological process

(Jackson and Busch, 1991; Casu and Lindahl. 2001; Kariya *et al*., 1997). Example of sulphated GAGs are chondroitin sulphate, dermatan sulphate, keratin sulphate, heparin sulphate and heparin (Masre *et al*., 2012). Sulphated GAGs play an important role in anti-coagulation (Kariya *et al*., 1997), pathology of amyloid disease such as amyloid A-amyloidosis, Alzheimer's disease and Parkinson's disease (Masre *et al*., 2012), studied the content of sulphated GAGs on three different anatomical regions (integument, internal organs and coelomic fluid) of *S. hermanii* and *S. vastus*. The integument showed the highest content of total sulphated GAGs for both *S hermanii* and *S. vastus* protein respectively (Masre *et al*., 2012).

According to Fredalina et al. 1999, fatty acid content in sea cucumber lipids fractions are the most important element, responsible for tissue repair and wound healing properties of this marine animal. Arachidonoic acid (C20:4 n-6) in Polyunsaturated fatty acids (PUFA) group was found to be the major component in almost all species of sea cucumber especially in tropical region (Svetashe *et al*., 1991; Drazen *et al*., 2008). It is reported to play a potential role in growth and blood clotting process leading to wound healing (Gil, 2002). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that found in several species of tropical and abyssal sea cucumbers is medically significant as these two long chain fatty acids are related with the reduced incidence of coronary heart disease and certain cancers (Roynette, 2004).

A number of published reports have revealed that some of sea cucumber species contain several potentially antiproliferative compounds. Research done by Althunibat *et al*., 2009 on antiproliferative activities of three Malaysian sea cucumber species

demonstrated that *Stichopus chloronotus* exhibited anti-proliferative effects against the cancer cells (Althunibat *et al*., 2009). Interestingly, *Stichopus chloronotus* aqueous extract was more toxic against C33A cells (human cervical cancer) compare to A549 (human non-small lung carcinoma cancer cells). *Stichopus chloronatus* organic extract was highly cytotoxic against C33A cells l but less activity against A549.

High molecular fraction of hot water extracts from sea cucumber, *Stichopus japonicus* inhibited the growth of human colon adenocarcinoma cells in a dose dependant manner (Ogushi *et al*., 2005). Ogushi *et al*., (2006) also conclude that the observed decreased in the growth of heterogeneous human epithelial colorectal adernocarcinoma (Caco-2) cells further exposure to *S. japonicus* extract resulted from induction of apoptosis (Ogushi *et al*., 2006). A number of important bioactive compounds identified in *Stichopu*s species are given in Table 1.1

Table 1.1: Medicinally important bioactive compounds in different species of sea cucumbers.

Bioactive compound	Sea cucumber species	Reference
Glycosaminoglycan	*Stichopus japonicus* *Stichopus vastus* *Stichopus hermanii*	(Kariya *et al*., 1997; Masre *et al*., 2012)
Lectin	*Stichopus japonicus*	(Himeshima *et al*., 1994;Matsui *et al*., 1994; Hatakeyama *et al*., 1993)

Bioactive peptides (protein gelatin & collagen hydrolysates)	*Stichopus japonicus*	(Saito *et al*., 2002; Ohtani *et al*., 2002; Birenheide *et al*., 1998)
Phenol and flavonoids	*Stichopus chloronatus*	(Althunibat *et al*., 2009)
Mucopolysacharides (SJAMP)	*Stichopus japonicus*	(Lu and Wang, 2009)
Polyunsaturated fatty acids (PUFA): arachidonic acid (AA C20:4 n-6) Eicosanpentaenoic acid (EPA C20:5 n-3) Docosahexaenoic acid (DHA C22:6 n-3)	*Stichopus hermanii* *Stichopus chloronatus*	(Fredalina *et al*., 1999; Svetashev *et al*., 1991)

1.2 Cancer

Cancer arises from normal cells through genetic alterations affecting the strongly controlled systems for growth control. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected (Hanahan and Weinberg, 2000). A mutation usually followed by an accelerated cellular proliferation resulting in increasing mass of cells (Vogelstein and Kinzler, 1993). Cancer symptoms vary widely based on the type of cancer. Cancer treatment may include chemotherapy, radiation, and/or surgery.

Cancer is mostly classified according to its organ, or cell type in which its starts. Also, it can be classified into wider categories are:

- Carcinoma – cancer of epithelial cells which initiates in tissue or skin that line or cover body organs
- Sarcoma – cancer of the connective tissues, like cancer that begins in blood, muscle, bone, cartilage, fat or other supportive or connective tissues.
- Lymphoma and myeloma – cancers that develop from cells originated from immune system
- Central nervous system cancers – cancers that occur in tissues of the central nervous system, such as brain and spinal cord
- Leukaemia – cancer that produces a large number of abnormal blood cells in blood generating tissue, such as bone marrow (Montella *et al*., 2001; Reya *et al*., 2001).

1.2.1 Invasion and Metastasis

A malignant tumor is a group of cancer cells that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body (Salehi *et al*., 2008). Invasion refers to the direct migration and penetration by cancer cells into neighboring tissues. Metastasis refers to the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade normal tissues elsewhere in the body. This ability of tumor cells metastasize is the cause of 90% of human cancer death (Sporn and Suh, 2000). Figure 1.3 shows the cancer cells invade through the basement membrane of the breast duct they can colonize the blood and lymphatic

vessels of the breast and migrate to other parts of the body. Invasion requires extracellular matrix adherence, destruction of protein in the matrix and stroma and motility. Metastasizing cells can enter via the lymphatics, or directly enter the circulation.

Few classes of proteins involved in the tethering of cells to their surrounding in a tissue are altered in cells possessing invasive or metastatic capabilities. Cancer cells motility involves integrin signaling, focal contact formation and actomysin-dependent contractility. Matrix Metalloproteinase (MMPs) and cathepsins are Extra Cellular Matrix (ECM) degrading enzyme that frequently up regulated in tumor cells and helps in cells cancer migration (Friedl and Wolf, 2003; Condeelis et al., 2005; Hanahan and Weinberg, 2011). MMPs are capable of cleaving all the extracellular matrix components of the parenchymal and vascular basement membrane, mechanical barriers to cell migration and invasion (Yi Tang, 2005).

There are at least three important steps involved in the process of tumor invasion . First is the attachment to the extracellular matrix (ECM), which may be mediated by pre-existing or newly formed contact sites. Second, is the creation of a proteolytic defect in the ECM and the final phase is migration through the proteolytically modified matrix (Ray, 1994). Among several MMPs that are involved in cancer progression, Gelatinase-A (MMP-2) and Gelatinase-B (MMP-9) are the most important key. MMP-2 is expressed in very early stage of breast cancer and contributes to the first leading of tumor formation (Poulsom *et al*., 1993). Meanwhile, MMP-9 also highly expressed in human breast carcinoma tissue and appears to be associated with lymph node metastasis

(Iwata *et al*., 1996). Few other studies also has been conducted to verify significance of MMP-2 and MMP-9 expression in breast cancer progression (Rha *et al*., 1997; Stetler-Stevenson, 1996; Remaele *et al*., 1993).

Cancer therapeutics that are designed to target adhesion receptors or proteases have not proven to be effective in slowing tumor progression in clinical trials, this might be due to the fact that cancer cells can modify their migration mechanisms in response to different conditions (Friedl and Wolf, 2003).

1.3 Breast cancer

1.3.1 Statistic

Breast cancer is the most frequently diagnosed cancer among women all over the world (Salehi *et al*., 2008; Cheng *et al*., 2006). Earlier detection and improved therapy have decrease the mortality rates of breast cancer patients but it still on the second ranking as women's killer after lung cancer (DeBruin and Josephy, 2002). Data from the National Cancer Registry of Malaysia for 2006 provide an age standardized incidence rate (ASR) 0f 39.9 per 100 000 women.

1.3.2 Etiology

Breast cancer is a malignant tumor that starts in the cells of the breast. Breast cancer originates from different types of cells in the breast. The breast is composed of a ductal system (milk ducts), lobules (milk sacs), fat, and connective tissue (tissue that supports the breast). Figure 1.4 shows that most breast cancers originate from the milk ducts (invasive ductal carcinoma, ductal carcinoma in situ) or from the milk lobules as

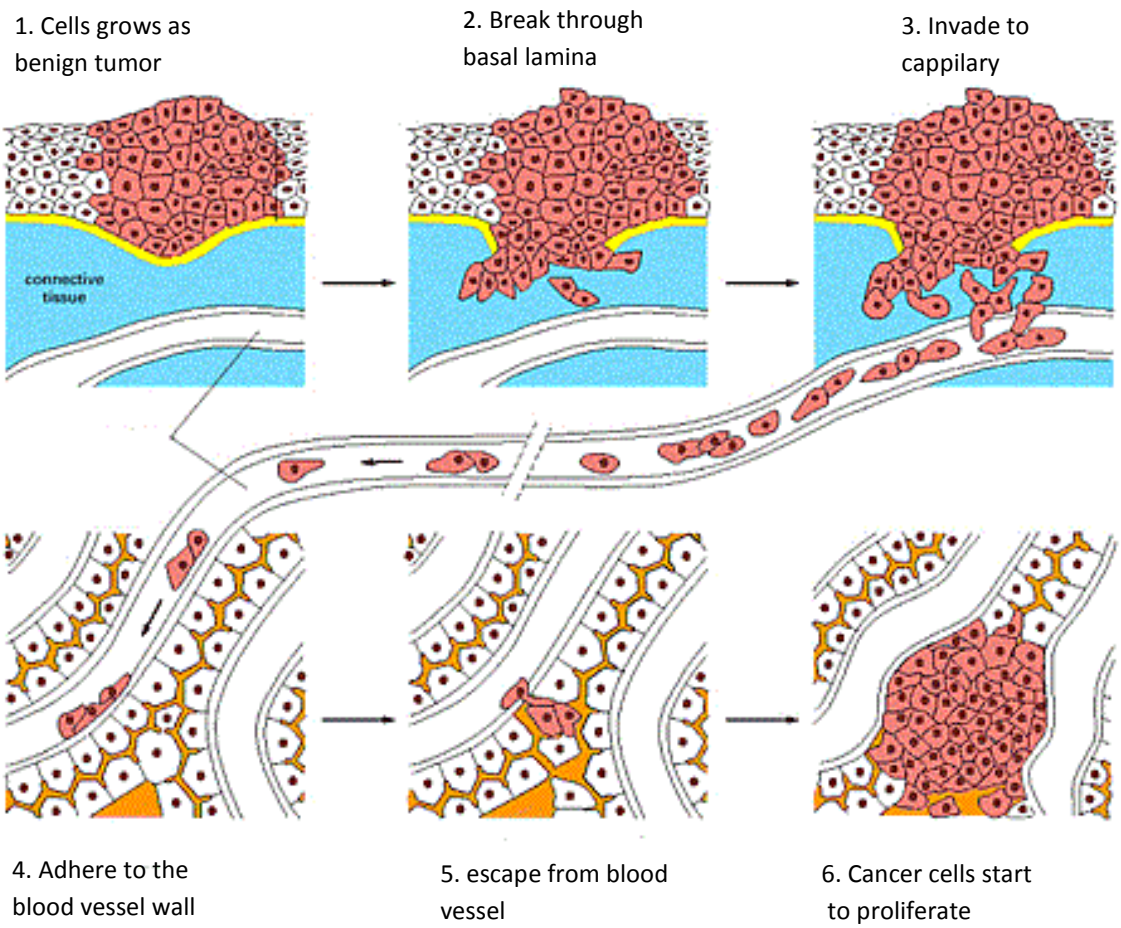

Figure 1.3: A schematic diagram shows the process of invasion and metastasis in cancer development [electronic print] Available at: www.cancer.gov [Accessed 2 April 2013].

invasive lobular carcinoma (McSherry *et al*., 2007)**.** Figure 1.5 shows in 2006, breast cancer was the most common cancer among female and also the most important cancer among population regardless of sex in Peninsular Malaysia. There were 3525 female breast cancer cases registed in National Cancer Institute (NCR) for that year, accounted for 16.5% of all cancer registered.

Numerous etiologic factors have been suspected and can be grouped into three broad factors; hereditary factor, hormonal and reproductive factor and environmental (including lifestyle) factor (DeBruin and Josephy, 2002). There are two genes that are associated with breast cancer hereditary, BRCA1 which is identified on chromosome 17 and BRCA2 on chromosome 13. When individual carry a mutated form of either BRCA1 or BCRA2, they have an increased risk of developing breast cancer at some point of their lives (Petrucelli and Feldman, 1998).

Recently studied done by Freday (2004) showed that environmental factor is the dominant factor compare to other factors in increasing breast cancer incidence. The changing of lifestyle pattern certainly contributed to higher risk of incidence and mortality. Developing countries shows rapid increase in breast cancer incidence, consequence of westernization of lifestyle, childbearing, dietary habit, and exposure to exogenous estrogen (Bray *et al*., 2004).

1.3.3 Estrogen and breast carcinogenesis

The relationship of breast cancer and estrogen has been known for more than hundred years after George Beatson revealed that removal of ovary can decrease the risk of

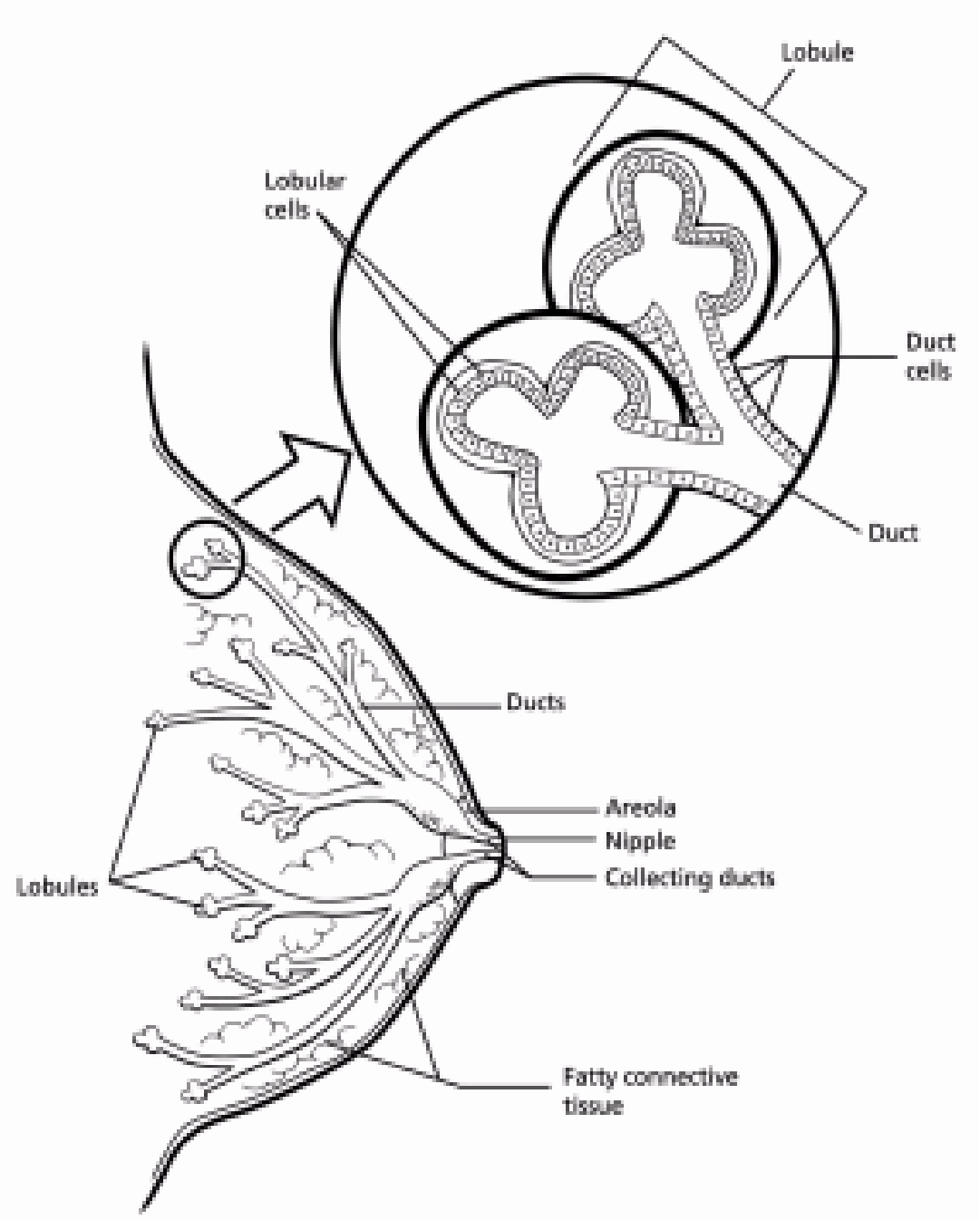

Figure 1.4: The illustration of the breast cancer origin during early stage of breast cancer development. [electronic print] Available at: www.cancer.org/cancer /breastcancer /detailedguide/ breast-cancer-what-is-breast-cancer [Accessed 21 April 2013].

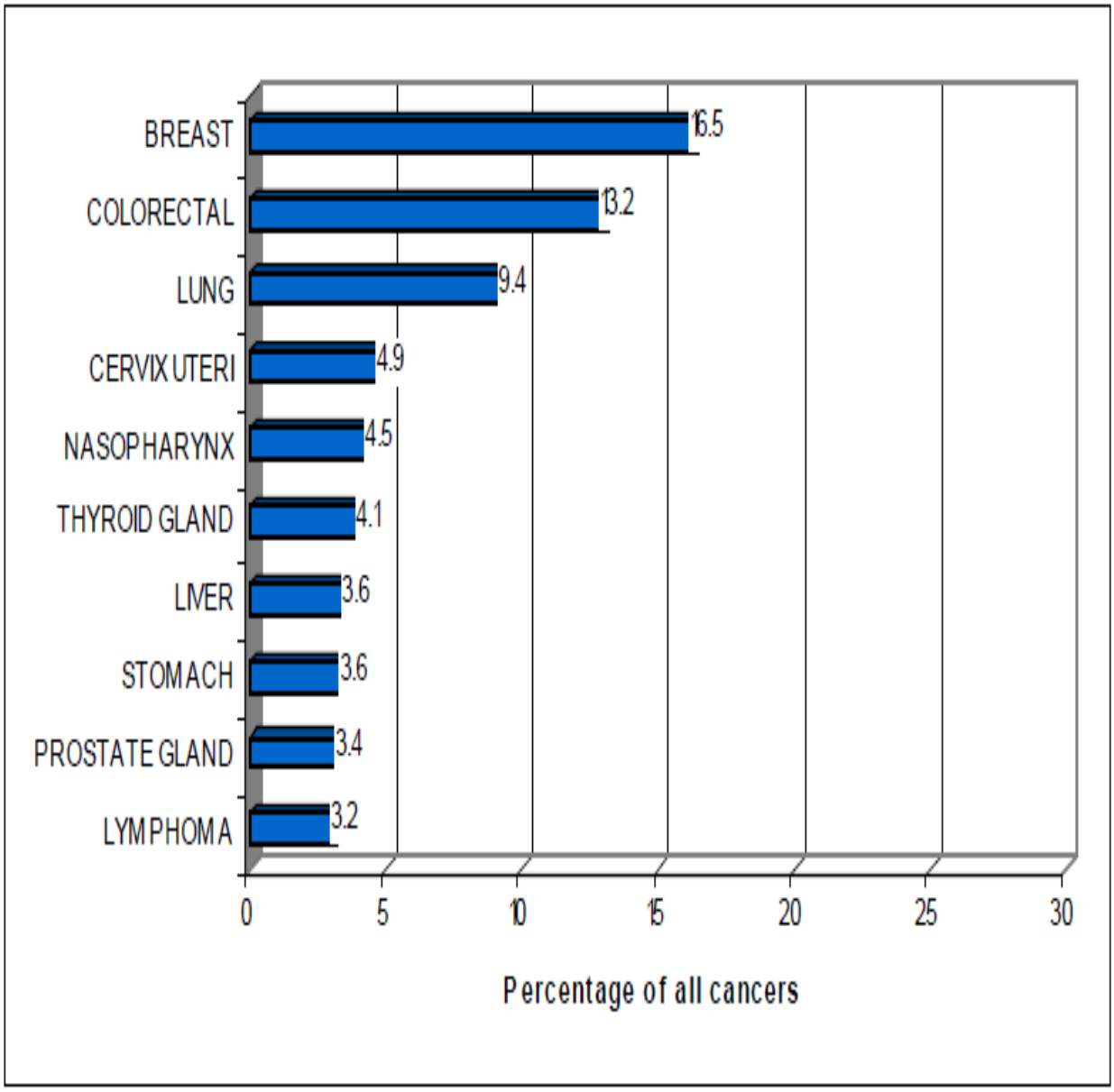

Figure 1.5: Ten most frequent cancer in Peninsular Malaysia (2006) (Adapted from: National Cancer Registry Malaysia, 2006)

getting a breast cancer (Beatson, 1896). Estrogen are steroid hormones that interact with intracellular estrogen receptor ERα and ERβ to activate gene transcription via estrogen response elements located near the promoter regions of estrogen responsive genes. Relative expression of α to β receptor is higher in invasive tumors in comparison to normal breast tissue. The balance between the receptor is important in determining the sensitivity of tissue to estrogen and thus the relative risk of breast carcinogenesis. In vitro, α and β receptors form heterodimers with each other and β receptor decrease the sensitivity of the α receptor. Thereby, receptor β can also acts as physiological regulator of the proliferative effects of α receptor (Hall and McDonnell, 1999; Leygue *et al*., 1998; Mark and Goss, 2009; Soommer and Suzanne, 2001).

A lot of experimental data strongly suggest that estrogens not only have a role in the growth of normal mammary epithelia tissue but also involved in the development of breast cancer (Lupulescu, 1995). Estrogen promotes the development of mammary cancer both direct and indirect proliferative effect on cultures breast cancer cells from humans. Direct tumor initiating effects may occur through the induction of enzymes and protein involved in nuclei acid synthesis. Indirect effect is through the stimulation of prolactin secretion and production of growth factor (Henderson *et al*., 1982; Pike *et al*., 1993).

1.4 Mechanism of Cell Death

Cell deaths are often defined as irreversible phase or 'point of no return. In 2005, The Nomenclature Committee on Cell Death (NCCD) has proposed that a cell should be considered dead when three following morphological criteria are met. Firstly, the cell

has lost the integrity of its plasma membrane, as defined by the incorporation of vital dyes, example propodium iodide. Second, the cell including its nucleus has undergone complete fragmentation into discrete bodies or frequently referred as apoptotic bodies and a last criterion is when the fragments have been engulfed by an adjacent cell in vivo (Kroemer *et al*., 2009).

1.4.1 Functions and types of cell death

The death of a cell can be defined as an irreversible loss of plasma membrane integrity and can be classified according to its morphological appearance which is apoptotic, necrotic and autophagy. Morphologically, apoptosis are cells that undergo shrinkage and blebbing of cells, rounding and blebbing of nuclei with condensation and margination of chromatin, cleavage of chromosomal DNA and packaging of the deceased cell into apoptotic bodies without plasma membrane breakdown (Kerr *et al*., 1994; Kerr *et al*., 1972). Apoptotic bodies are recognized for the absence of inflammation around the dying cell hence been removed by phagocytic cells .

Necrosis is the name given to unprogrammed death of cells and living tissue. It been characterized by gain in cell volume, enlargement of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Kroemer *et al*., 2009). In contrast with apoptosis, liberation of factors from dead cells will alert the innate immune system and triggers the local inflammation. The lack of signaling from necrotic cells make it more complicated for nearby phagocytes to engulf the dying cells hence contributes to the inflammation response. Necrosis may be caused by inflammation, cancer, infection, infarction, toxin and injury.

Autophagy is a cell that goes through nutrient deprivation and change to the stationary phase that allows survival in less complex environment (Eisenberg-Lenner *et a*l., 2009). The breakdown of cellular components can help cellular survival during starvation by maintaining cellular energy level (Baehrecke, 2005). This type of cell death also known as a type of cell death that happen in the absence of chromatin condensation but parallel with autophagic vacuolization of the cytoplasm and not involving the (Clarke, 1990). Autophagy has been observed to enable continued growth of tumor cells by reduced the cellular energy usage in order to maintain their energy. Inhibiting the genes of autophagy in tumor cells will lead to extended survival of the organs affected by the. Furthermore, inhibition of autophagy also revealed that it can enhance the effectiveness of cancer therapies (Curvo, 2004; Levine and Klionsky, 2004; Lockshin and Zakeri, 2004).

1.4.2 Apoptosis and cancer

Apoptosis characteristically affects scattered cells, not group of adjoining cells (Kerr *et al*., 1994). Morphologically, cells that undergo apoptosis will face few criteria that differentiate between necrosis and apoptosis, for example; shrinkage and blebbing of cells, rounding and blebbing of nuclei with condensation and margination of chromatin, slight shrinkage or morphologically undetectable changes in organelles, and phagocytosis of cell fragments without accompanying inflammatory response (Lockshin and Zakeri, 2004). Figure 1.6 illustrating sequence of ultra-structural changes in apoptosis (2-6) and necrosis (7-8). There are two major mechanism of cell death (necrosis and apoptosis). Cells that are damaged by external injury undergo necrosis,

while cells that are induced to commit programmed suicide because of internal or external stimuli undergo apoptosis.

The apoptosis cascade includes two major pathway involving extrinsic pathway and intrinsic pathway. Extrinsic pathway involving activation of death receptors in response to ligand binding or intrinsic pathway which is release of proapoptotic proteins, for example cytochrome C from mitochondria. The major player for both pathways is the caspases which also function as the executioner in apoptotic event (Green and Reed, 1998).

The activation of caspases in the mitochondrial pathway requires mitochondrial outer membrane permeabilization (Chipuk *et al*., 2006) . Cytochrome C will be release form mitochondria and bind to apoptotic protease activating factor 1 (APAF1). This event directly leading to the activation of platform called apoptosome. Apoptosome subsequently will activates the initiator caspase (caspase 9) which will cleaves and activates caspase 3 and 7 (Stephen and Douglas, 2010). The extrinsic pathway, involve death receptor ligation causes the recruitment of adaptor molecules such as FAS-associated death domain protein FADD, that bind dimerize and activate caspase 8. As what happened at intrinsic pathway, capase 8 also directly cleaves and activates caspase 3 and 7 (Askenazi and Dixit, 1998).

Cells that undergo apoptosis in response to intrinsic stimuli have the permeability of the mitochondrial outer membrane that assist by activation of two essential protein, BCL-2 associated X protein (BAX) or BCL-2 antagonist or killer (BAK) (Kroemer, 1997;

Fernhead *et a*l., 1998; Soengas *et al*., 1999). In order for a cancer cells to be resistance to apoptosis, the cells will expressed the anti-apoptotic protein such as Bcl-2 or by the downregulation or mutation of pro-apoptotic protein such as Bax. The expression of both Bcl-2 and Bax are regulated by the product of the p53 tumor suppressor gene. p53 is the most important checkpoint that involved protein in cell cycle arrest and maintaining genomic integrity following DNA damage. Therefore, p53 have been known as one of the unique molecular target for cancer therapy (Sun and Peng, 2012; Chipuk *et al*., 2006).

1.5 Cell migration

1.5.1 Cell migration and wound healing

Cell migration is an essential part of most normal biological processes and also many diseases. Cell movement is a complicated and tightly controlled process, consists of arrangement of variety of different proteins (Kristy and Lynch, 1993; Bartold and Raben, 1996). Figure 1.7 illustrate the process of cell migration. In the beginning steps, the front part of the cells is moving forward response to the extension of the internal cytoskeleton. Lamellipodium or a flat structure will form as a result of the movement of the cell edge. Adhesion molecules play an important role by attached the leading edge of the cell and allows the cell to pull the rest of the cell body forwards. Finally the cell detaches the back of the cell - often termed the trailing edge - and pulls it back into the main cell body. This process will be repeated as as the cell continues to move forward. The migration of the cells also involved in vascular disease, chronic inflammation, cancer and virus bacterial infection (Schafer and Werner, 2008).

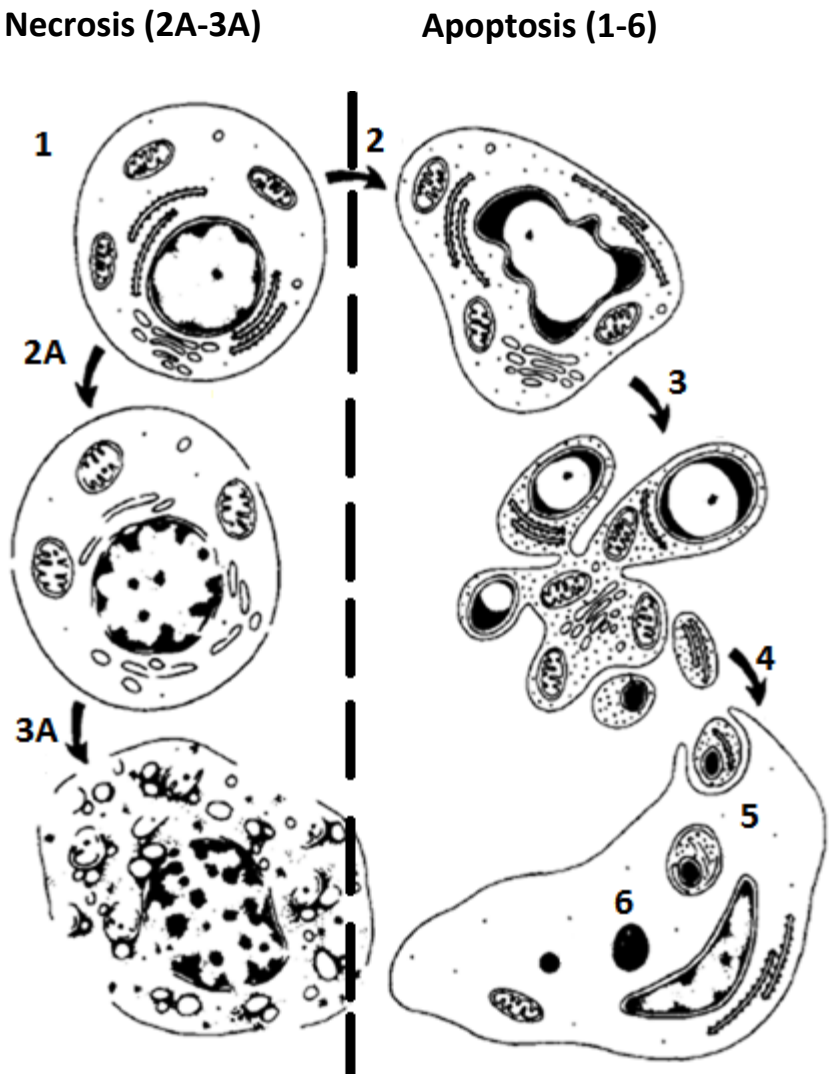

Figure 1.6: Diagram of structural changes of cells undergoing apoptosis (1-6) and necrosis (2A-3A). (1) Normal cell. Early apoptosis (2) is characterized by campaction and margination of nuclear chromatin, condensation of cytoplasma and convolution of nuclear and cell outlines. (3) At a later stage, the nucleus fragments, and proturberances that form on the cell surface separate to produce apoptotic bodies, which (4) are phagocytosed by nearby cells and (5 and 6) degraded with lysosomes. (2A) The development of necrosis is associated with irregular clumping of chromatin, marked swelling of organelles and focal disruption of membranes. (3A) Membranes subsequently disintegrate, but the usually retains its overall shape until removed by mononuclear phagocytes. (Adapted from: Apoptosis Its significance in cancer and cancer therapy.)

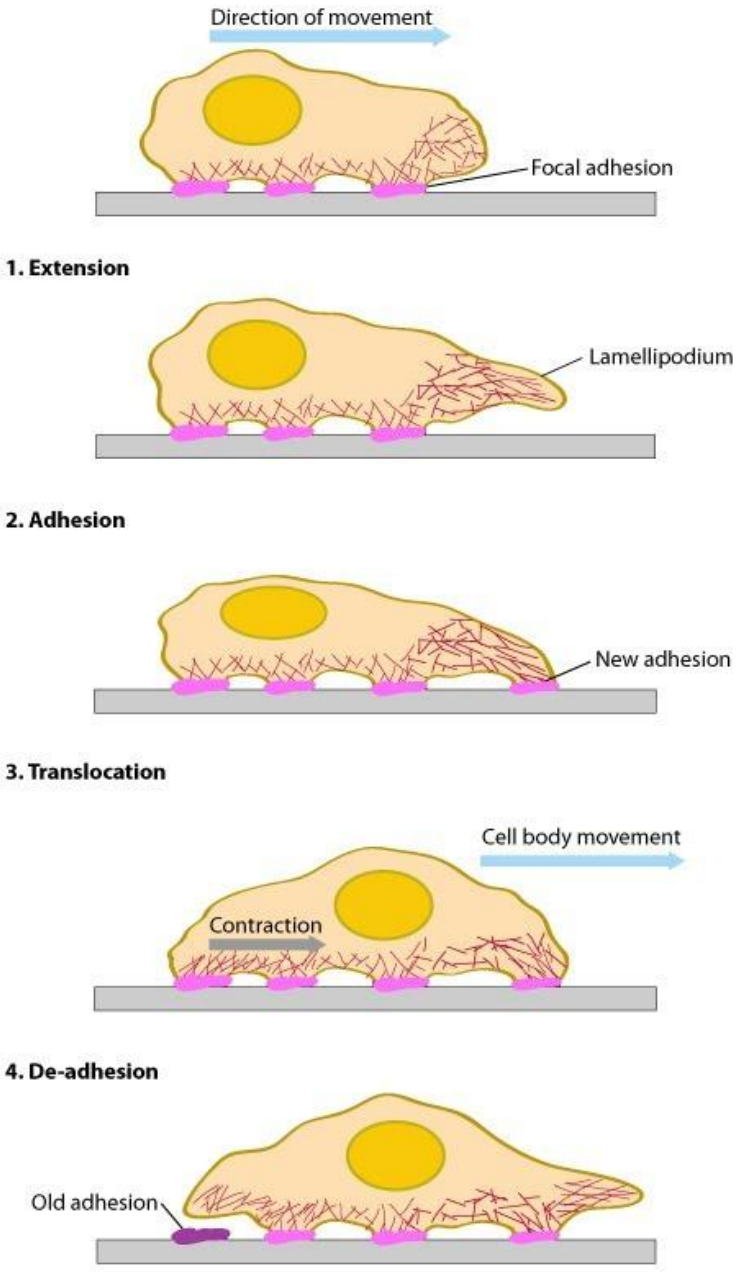

Figure 1.7 : Conserved steps in cell spreading and movement. (1) Polymerization of actin filaments at the leading edgeis translated into protrusive force. (2) Membrane protrusion facilitates the binding of transmembrane cell surface receptors to the substratum components. New adhesions are rapidly linked to the network of actin filaments. (3) The combined activity of retrograde actin movement and contractile forces produced by stress fibers generate tension to pull the cell body forward. (4)The forces produced by the contractile network combined with actin filament and focal adhesion disassembly helps to retract the trailing cell edge.

1.5.2 Fibroblast and wound healing

A fibroblast cell is one of variety of cells that were known to be involved in wound healing process. It is a type of cell that synthesizes and degrades the ECM components by expressing collagen, the structural framework (stroma) for animal tissues, and plays a major role in wound healing (Marsh *et al*., 2012; Jacob and Pure, 2012). Fibroblasts are play an important role in preparing the suitable condition for wound healing process by providing basic structural integrity and produce the tissue specific basement membranes that provide a protective barrier around the specialized epithelium (Montella *et al*., 2001).

Wound healing consists of three phases, inflammation, tissue formation and tissue remodeling (Witte and Barbul, 1997). Inflammatory cells take place right after injury, the inflammatory cells attract to the wound site for defense against invading bacteria. Inflammatory cells also important for generate growth factor, cytokines and proteinases which important for development of new tissue (Reya *et al*., 2001). Repairing of the injury take place with the beginning of re-epitheliazation phase by new epidermis. Fibroblast migrate into the wound, where they proliferate and produce large amounts of extracellular matrix (ECM) necessary to support cell growth and blood vessel carry oxygen and nutrient necessary to sustain cell metabolism (Hanahan and Weinberg, 2000). On the contrary, the extracellular matrix can have a good or bad consequence on the ability of fibroblast to synthesize, deposit, remodel and generally interact with the extra cellular matrix. Myofibroblasts are generated form fibroblast and responsible for wound contraction and the deposition of additional matrix. It also plays an important role in wound healing process. Growth factors also one of the key factor in wound

closure process in stimulating fibroblast of the tissue around the wound area to proliferate, express appropriate integrin receptor and migrate into wounds space to treat chronic pressure sore (Vogelstein and Kienzler, 1993).

After moving into the wound, fibroblast begins the synthesis of extracellular matrix. The temporary extracellular matrix is gradually switch places with the collagenous matrix. When enough collagen matrix has been transferred in the wound area, the fibroblast will stop constructing collagen, and the fibroblast rich granulation tissue is substituted by a cellular scar (Marsh, *et al*., 2012).

1.6 Aims of the Thesis

Although 'Gamat' extracts is locally appreciated for its well purported mysterious efficiency and very minimal documented side effects, there are still however very insufficient evidences in validating their efficiencies and mechanism. There are only a few pharmacological studies on the potential of the extracts from this species discussing the efficacy and it bioactivity potential

Therefore, this study aimed to:

1. To extract sea cucumber, *Stichopus horrens* and *Stichopus vastus* using organic and aqueous extraction methods.
2. To determine the cytotoxicity of *S. horrens* and *S. vastus* extracts
3. To investigate whether *S. horrens* and *S. vastus* have the ability to inhibit the invasion of breast cancer and potential in wound healing properties