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IN-VITRO ANTI-CANCER STUDIES OF *ARCTIUM LAPPA* L. EXTRACT

MOHAMAD TALEB AGHA

UNIVERSITI SAINS MALAYSIA

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IN-VITRO ANTI-CANCER STUDIES OF *ARCTIUM LAPPA* L. EXTRACT

by

MOHAMAD TALEB AGHA

Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science (Pharmacology)

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This thesis is dedicated to …

My parents,

My brother and sisters

And People of Syria

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
A. lappa	*Arctium lappa L.*
Ang-1	Angiopoietins-1
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bcl-1 and 2	B-cell lymphoma 1 and 2
bFGF	basic Fibroblast growth factor
c-myc	Myelocytomatosis oncogene cellular homolog
CO_2	Carbon dioxide
Conc	Concentration
3D	Three-dimensional
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

DPPH	1,1-Diphenyl-2-picrylhydrazyl
e.g.	For example
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen receptor
etc.	Et cetera, it means “and other things”
FTIR	Fourier transform infrared spectrometry
GA	Gallic acid
GAE	Gallic acid equivalent
GC-MS	Gas chromatography mass spectrometry
GTP	Guanosine triphosphate
HCT-116	Human colorectal carcinoma cell line
HIFBS	Heat-inactivated fetal bovine serum
HIFs	Hypoxia inducible factors
i.e.	That means
IC_{50}	Half-maximal inhibitory concentration
IL	Interleukin
IFN	Interferon

IR	Infrared
M199	Earle's salt
MCF-7	Human hormone sensitive and invasive breast cancer
MDA-MB-231	Human hormone resistant breast cancer cell line
MIF	Migratory inhibitory factor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide
mRNA	Messenger Ribonucleic acid
m/z	Mass/charge ratio
NO	Nitric oxide
OD	Optical density
ORP150	Oxygen-regulated protein 150
p53	Tumor suppressor protein 53
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PE	Plating efficiency
PlGF	Placenta growth factor
PS	Penicillin/streptomycin solution

RB	Retinoblastoma protein
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute medium
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SF	Surviving fraction
SPSS	Statistical Package for the Social Sciences
TEM	Transmission electron microscopy
Temp	Temperature
TGF-β	Transforming growth factor- β
TMX	Tamoxifen
TNF	Tumor necrosis factor
TSP-1	Thrombospondin-1
USA	United states of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
UV-Vis	Ultraviolet visible

VEGF	Vascular endothelial growth factor
VEGFRs	Vascular endothelial growth factor receptor
vs.	Versus
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/ weight

LIST OF UNITS

Cm	Centimeter
G	Gram
H	Hour
Kg	Kilogram
Mg	Milligram
Ml	Milliliter
Mm	Millimeter
mm^3	Cubic millimeter
Min	Minute
Pg	Picogram
U	Unit
Mg	Microgram
µl	Micro litter
M	Micron

LIST OF SYMBOLS

A	Alpha
B	Beta
E	Epsilon
≈	Approximately equal to
ºC	Degree Celsius
+ve	Positive
–ve	Negative
%	Percent

KAJIAN ANTI KANSER *IN VITRO* UNTUK EKSTRAK *ARCTIUM LAPPA* L.

ABSTRAK

Kanser payudara adalah kanser kedua paling lazim dilaporkan dan penyebab kelima kematian di seluruh dunia. Kanser ini dikelaskan kepada dua jenis; reseptor-estrogen (ER) negatif dan ER positif. Rawatan kanser payudara merangkumi pembedahan, kimia atau gabungan kedua-duanya. *Arctium lappa* L., sejenis rumpai yang boleh dimakan berasal dari Eropah Utara, dengan taburan sederhana di seluruh Asia dan Amerika, telah digunakan secara tradisional untuk merawat kanser, diabetes dan keradangan. Dalam kajian ini, asai MTT telah dijalankan untuk mengkaji potensi antikanser ekstrak dan fraksi *A. lappa* menggunakan jujukan sel kanser payudara hormon-sensitif (MCF-7), jujukan sel kanser kolorektal (HCT-116) dan jujukan sel normal (EA.hy926). MCF-7 didapati sangat sensitif terhadap ekstrak-ekstrak *A. lappa*, dengan ekstrak etanolik menunjukkan kesan yang paling aktif. Fraksi n-heksana bagi ekstrak etanolik (EHX) menunjukkan aktiviti yang kuat terhadap kedua-dua jujukan sel MCF-7 dan EA.hy926 (IC_{50}: 14.08 ± 3.64 and 27.25 ± 3.45 μg/ml, masing-masing). Perubahan morfologi di dalam jujukan sel MCF-7 yang dirawat telah dikaji menggunakan teknik mikroskop elektron transmisi. Kesan apoptosis yang jelas dikesan. Tambahan pula, EHX didapati menganggu proses metastatik sel kanser payudara melalui perencatan percambahan, migrasi, invasi dan kolonisasi sel. Kesan-kesan EHX terhadap 10 laluan isyarat kanser telah dikaji. Fraksi menaikkan regulasi p53, β TGF dan NFkB ($p < 0.05$) secara signifikan. Seterusnya, sifat-sifat antiangiogenik daripada EHX dinilai menggunakan model-model *ex vivo* dan *in vitro*. EHX telah menunjukkan kesan antiangiogenik yang mujarab kerana ia merencat sebahagian besar percambahan mikro

kapal tikus aorta (IC_{50} 4.34 ± 1.64 µg / ml). Kajian *in vitro* selanjutnya menunjukkan EHX merencat migrasi dan kolonisasi sel-sel EA.hy926 dengan berkesan. Sebagai contoh, EHX pada kepekatan 14 µg/ml merencat pembezaan sel-sel endotelial dan pembentukkan salur baru sebanyak 93%. Ekspresi faktor pertumbuhan endotelial vaskular (VEGF) didapati berkurangan sebanyak 54% di dalam sel-sel kanser yang dirawat dengan 30 µg/ml EHX. Analisis fitokimia EHX menunjukkan kandungan fenolik dan flavonoid yang lebih tinggi daripada ekstrak induk, dengan aktiviti antioksida yang besar. Lebih 20 kompaun telah dikenal pasti di dalam EHX, yang mana stigmasterol, beta-sitosterol, dan 3-O-acetyllupeol adalah kompaun utama. Kesimpulannya, kajian ini menunjukkan *A. lappa* mempunyai aktiviti antikanser dan antiangiogenik yang mungkin berguna di dalam rawatan kanser payudara.

IN-VITRO ANTI-CANCER STUDIES OF *ARCTIUM LAPPA* L. EXTRACT

ABSTRACT

Breast cancer is the second most common cancer and the fifth cause of death worldwide. It is classified into two types: estrogen-receptor (ER) negative and ER positive. Treatment of breast cancer could be surgical, chemical or a combination of both. *Arctium lappa* L., an edible weed indigenous to Northern Europe, with moderate representation across Asia and America, has been traditionally used to treat cancer, diabetes and inflammation. In this work, *in vitro* studies were conducted to investigate the anticancer potential of *A. lappa* extracts and fractions using a hormone-sensitive breast cancer cell line (MCF-7), a colorectal cancer cell line (HCT-116) and a normal cell line (EA.hy926). MCF-7 was found to be highly sensitive to *A. lappa* extracts, with the ethanolic extract being the most active. The n-hexane fraction of the ethanolic extract (EHX) showed strong activity against both MCF-7 and EA.hy926 cell lines (IC_{50}: 14.08 ± 3.64 and 27.25 ± 3.45 μg/ml, respectively). Morphological alterations in treated MCF-7 were visualized using transmission electron microscopy techniques. Distinct apoptosis was detected. Furthermore, EHX was found to disrupt the metastatic cascade of breast cancer cells by inhibition of cell proliferation, migration, invasion and colonization. Effects of EHX on 10 major cancer pathways were also investigated. The fraction was found to significantly up-regulate p53, TGF β and NFkB ($p < 0.05$). Antiangiogenic properties of EHX were assessed using *ex vivo* and *in vitro* models. EHX was shown to be potently antiangiogenic as it substantially repressed the sprouting of micro-vessels in rat aorta (IC_{50} 4.34 ± 1.64 μg/ml). Further *in vitro* studies showed EHX to strongly inhibit the migration and colonization of EA.hy926 cells. For At 14

µg/ml of EHX suppressed the differentiation of endothelial cells and the formation of neovascularization by 93%. The expression of the vascular endothelial growth factor (VEGF) was found to be down-regulated by 54% in cancer cells treated with 30 µg/ml of EHX. Phytochemical analysis of EHX showed higher phenolic and flavonoid contents than the parent extract, with a substantial antioxidant activity. Over 20 compounds were identified in EHX, of which stigmasterol, beta-sitosterol, and 3-O-acetyllupeol being the major active compounds. In conclusion, this work demonstrates that *A. lappa* has valuable anticancer activity and antiangiogenic properties that may be useful in breast cancer treatment.

CHAPTER ONE: INTRODUCTION

1.1 Cancer:

Cancer is a mass of tissue that occurs due to abnormal proliferation of cells which tend to metastasize to other distant body parts. Cancer is not one disease. It is a group of more than 100 different and distinctive diseases (Labuschagne et al., 2014).

Normally, human cells grow and divide to form new cells as and when the body needs them. When cells grow old or become damaged, they die, and new cells take their place. When cancer develops, this regulatory process disrupts. As cells become more and more abnormal, old or damaged cells still survive when they should die, and new cells form when they are not needed. These extra cells tend to proliferate non-stop and form a mass of tissue called tumor (Hartwell and Kastan, 1994).

Tumors are classified as cancerous tumors (malignant) or non-cancerous tumors (benign). Cancerous tumors means, the tumor cells are able to invade neighboring tissues and metastasize to other places in the body through the blood or the lymph system to form new tumors far from the primary. In contrast, benign tumors grow locally and do not spread out, and when surgically excised usually will not grow back, whereas malignant occasionally do (Khansur et al., 1987).

1.2 Cancer prevalence:

Cancer still has been recording a high number of incidence and mortality around the world. So far, cancer is still leading the second causes of death after heart diseases (Siegel et al., 2015). By 2012, we have witnessed 14.8 million new cases and 8.2 million deaths caused by different sorts of cancer. Lung cancer was diagnosed as the most common with 1.82 million cases followed by breast cancer 1.67 million cases then 1.36 million cases by colorectal cancer. The leading cause of cancer death were lung cancer (1.6 million deaths), followed by liver cancer (745,000 deaths), and stomach cancer (723,000 deaths) (Ferlay et al., 2015).

Males are more susceptible to cancer and have a higher rate of mortality comparing to females. Among men, the widespread sites of cancer cases in 2012 were lung, prostate, colorectal, stomach, and liver cancer. While among women, the 5 widespread sites were breast, colorectal, lung, cervix, and stomach cancer (Jemal et al., 2011).

In ASEAN region, the estimates were over 700,000 new cancer cases and 500,000 cancer deaths in 2008. The most commonly diagnosed cancers were lung (98,143), breast (86,842) and liver cancers (74,777). The leading cause of death was lung cancer (85,772), liver cancer (69,115) and colorectal cancer (44,280) (Kimman et al., 2012).

The National Cancer Institute in Malaysia reported that, 103,507 new incidence were diagnosed for the period 2007-2011. Whereas the most common cancer among Malaysian were breast at 17.7%, followed by colorectal 13.2%, and lung cancer 10.2% (National Cancer Institute, 2016).

Concluding, the rate of cancer mortality is higher in the less developed regions comparing to the developed due to lower quality of life and lack of health education (Ferlay et al., 2010).

1.3 Factors that have roles in cancer incidence:

Environmental carcinogens lead to abnormal proliferation of cells caused by gene mutation (Belpomme et al., 2007). The duration of exposure influences directly in increasing disease risk. Including, lifestyle, obesity, sun exposure, radiation, bacterial and viral pathogens (such as *Helicobacter pylori*, papilloma viruses, and hepatitis B or C virus) and chemical substances (such as tobacco, pesticides, asbestos, and industrial waste) (Wang and Chen, 2001). Some hormones are a key role in the development of cancers such as estrogen and testosterone in breast and prostate cancers (Cuzick, 2008).

Age, sex, race, heredity, lifestyle, pregnancy, sexual activity, and physiological stress also play a considerable role in cancer incidences (Sneden, 2004). Genetics plays significant contribution towards cancer cases; classified as two classes of genes: proto-oncogenes (stimulate growth of cancer cells) and tumor suppressor genes (stop the cell division mechanism and repress tumor growth), as shown in Table 1.1 (Weeraratna, 2005, Sneden, 2004).

Table 1.1 Some of oncogenes and tumor suppressor genes.

Oncogenes	**Cancer**	**Tumor suppressor genes**	**Cancer**
Abl	Leukemia	APC	Stomach and colon
Bcl-1	Breast	P53	Many cancers
c-myc	Leukemia, lung, and breast	VHL	Kidney
N-myc	Neuroblastoma	CDK4	Skin
BCR-ABL	Leukemia	DPC4	Pancreas
Ki-ras	Lung, colon, ovarian	BRCA1	Breast and ovarian
N-ras	Leukemia	BRCA2	Breast
CDKN2	Melanoma	MSH2 and MSH6	Colon
MDM2	Sarcoma	MLH1	Colon
PDGF	Glioma	RB	Many cancers
Erb-B	Breast		
HPC1	Prostate		
RET	Thyroid		

1.4 Classification of cancer types:

Cancer can be classified according to the following:

1.4.1 Tissue type:

There are three different types described as follow: carcinoma, sarcoma, and glioma. Carcinoma is widespread mostly where the tumor originates in the epithelial tissue. Sarcoma, when the tumor emerges in the connective tissue. Glioma is a tumor in non-neuronal brain tissue (Yarbro et al., 2010).

1.4.2 Targeted organs:

Cancer can attack almost all organs in the body with different percentages of prevalence; starting from skin, blood (leukemia), brain, breast, lung, pancreatic, colon, kidney, prostate, ovarian and uterine (Sneden, 2004).

1.5 Breast cancer:

Breast cancer is probably the world's first studied malignancy and it is a global priority. Research on the interplay between environment and genes has illuminated the workings of the disease and helped to identify who is really at risk (Woolston, 2015).

1.5.1 Epidemiology of breast cancer:

Breast cancer is relatively 100 times more common in women than men (Kreiter et al., 2014). It is the world's second most prevalent cancer with 10.9% of all cancers, but it is the fifth cancer type that leads to death. Globally, 1.7 million women were diagnosed with breast cancer in 2012. This disease kills around 400,000 women annually (Woolston, 2015, Ferlay et al., 2010, Formenti et al., 2011).

1.5.2 Types of breast cancer:

Two types of breast cancer can be defined in accordance to age, estrogen receptor (ER) negative such as MDA-MB-231 which is most common diagnosed before menopause with a serious and high rate of mortality. The ER positive such as MCF-7 is diagnosed after menopause and characterized by a lower mortality rate (Formenti et al., 2011). The estrogen-dependent breast cancer (ER positive) increases the aggressiveness of malignant tumor in this type of breast cancer which is dependent on hormonal effect. In contrast, activation of estrogen receptor of ER negative breast cancer reduces the seriousness (Sheikh et al., 1993).

1.6 Angiogenesis:

Since the 70s, much work have been carried out to find better cancer treatment and one of the many approaches includes the interception of tumor vasculature by disrupting its developmental stage, a process commonly identified as angiogenesis. Angiogenesis is a complicated process that plays considerable role physiologically in normal development of blood capillary, wound healing processes, embryonic development and for menstrual cycle including endothelial cell migration, colonization, proliferation and tube formation

(Lu et al., 2016, Folkman, 1971). But it is well established that unregulated angiogenesis participates in initiation and progression of many pathological states, such as obesity, cancer, inflammation, psoriasis and diabetic retinopathy. Malignant tumors generally tend to grow up larger than $1mm^3$ and that requires more oxygen and nutrients consuming. Hence building a new blood vessels network is crucial to support tumor growth by supplying their nutritional requirements. Without this network of capillary, tumors will not be able to survive and the metastasis of cancer cells to other localities will be definitely disrupted (Li et al., 2016, Eskens, 2004). Recently a number of antiangiogenic drugs have been approved for cancer therapy. These drugs are rated as adjuvants to standard chemotherapy regiments. One fine example is in the treatment of lung cancer where the combinational use of the chemotherapeutic agents carboplatin and paclitaxel with bevacizumab (antiangiogenic agent), which was shown to boost treatment efficacy. Other new developed antiangiogenic agents include cediranib, axitinib, aflibercept, nintedanib, pazopanib, vatalanib, brivanib, sorafenib, sunitinib, and ramucirumab (Shankar et al., 2016, Aggarwal et al., 2012, Medinger and Mross, 2010).

1.7 Role of VEGF in the regulation of angiogenesis:

VEGF vascular endothelial growth factor was discovered more than a decade ago and described as a major hypoxia-inducible growth factor (Li and Eriksson, 2001). VEGFs are pivotal factors for regulation of blood, lymphatic, and nervous systems throughout the pregnancy (embryonic development) and wound healing. In adults, VEGFs still play a role to retain homeostasis of vessels. Also, VEGFs mediate neovascularization

including several pathological impairments and diseases such as cancer (Ahmed and Bicknell, 2009).

VEGF is often referred to as VEGFA and is a member of bigger VEGFs family of growth factors. VEGFs family contains seven members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D VEGF-E, VEGF-F, and PLGF (placental growth factor). In addition, there are specific tyrosine kinase receptors (VEGFRs), including VEGFR-1, VEGFR-2 and VEGFR-3. These VEGFs differ in their expression pattern, receptor specificity and biological functions. VEGFA has been studied extensively comparing to other members of this family. VEGFA is a homodimer and it has eight distinguished variants (VEGF121, VEGF145, VEGF148, VEGF165, VEGF183, VEGF189, VEGF 110 and VEGF206) (Goel and Mercurio, 2013). It regulates blood vessel development and angiogenesis by binding to VEGFR-1 and VEGFR-2 receptors. VEGF-C and VEGF-D are activated by binding to VEGFR2 and VEGFR3 to control vascularization process during development and the lymphatic system in adults. VEGF-E is a specific factor in viruses and binds only to VEGFR-2 (Ahmed and Bicknell, 2009, Fong et al., 1995, Ferrara, 2001).

In cancer, it has become evident that the role of VEGF is not limited to angiogenesis and vascular permeability (Senger, 2010). VEGF, for example, has influence on the immune cells that are present in the tumor microenvironment and, consequently, it can affect the host response to tumors (Hansen et al., 2012). Moreover, VEGF

receptors may control the function of fibroblasts in the tumor stroma (Yaqoob et al., 2012). VEGF releasing is activated by hypoxic factor (HIF-1α) in hypoxic region. Whereas, VEGF transcription is stimulated by some oncogenes that activate HIF-1 expression; this increase tumor angiogenesis cascades (Ahmed and Bicknell, 2009).

Angiogenesis cascade is resembled as endothelial cells colonization, proliferation, migration, differentiation and survival activities and regulated by binding of VEGF to VEGFR-2 through VEGF receptor tyrosine kinase (RTKs) signaling pathway. The phosphorylation of tyrosine residue will be induced by VEGFs, to control the signals of VEGFR-2, whereas four autophosphorylation sites are identified by VEGFs (tyrosine's 951, 996, 1054 and 1059) (Ahmed and Bicknell, 2009, Dougher and Terman, 1999, Jussila and Alitalo, 2002). Later on, VEGF transphosphatidylation reaction will activate a mediatory enzyme phospholipase D, the function of this enzyme is to regulate angiogenesis phenotype, and this activation requires tyrosine phosphorylation and protein kinase C. It mediates many processes initiating angiogenesis pathways such as endothelial cell migration (Seymour et al., 1996).

VEGF also influences nitric oxide (NO) synthase, stimulates production of nitric oxide by mediation of VEGFR, this regulates sprouting of new blood vessels and increases endothelial cell proliferation and migration (Feliers et al., 2015).

1.8 Role of hypoxia in angiogenesis pathway:

Various epigenetic or environmental influences control neovascularization of malignant tumors and metastasis process like hypoxia, hypoglycemia, and acidosis. Hypoxia is a deprivation state of oxygen supply, in which there is an induction of tumor growth. HIFs hypoxia inducible factors are transcription factors activated when hypoxic region take place. Hypoxia-inducible factor 1 (HIF-1) is the most known among HIFs; its structure includes two DNA-binding proteins (heterodimer), HIF-1α and aryl hydrocarbon nuclear translocator (HIF-1β or ARNT) (Ahmed and Bicknell, 2009). In addition to HIF-1α, there are two more in HIFs family: HIF- 2α and HIF-3α. HIF-2α has less influence in normal adults and tumor angiogenesis in comparison to HIF-1α and HIF-3α, but it affects directly during pregnancy period (embryonic development) (Leek et al., 2002).

Hypoxia is a major driver for genetic expression of vascular endothelial growth factor (VEGF) (Goel and Mercurio, 2013). Moreover, other extra-cellular pathways also have substantial impact regarding to hypoxia induced angiogenesis include the ephrin/Eph receptor, roundabouts/slits, notch/delta, netrins/UNCs, angiogenin, endothelins 1 and 2, adrenomedullin, neuropillins, plexins, semaphorins, and a variety of intracellular protein families (hedgehog and sprout) (Ahmed and Bicknell, 2009, Bicknell and Harris, 2004). Furthermore, hypoxia regulates several other angiogenic pathways such as connective tissue growth factor (CTGF), which is a strong angiogenic factor in human breast cancer cells (Shimo et al., 2001); inducible endoplasmic reticulum oxygen-regulated protein 150 (ORP150), which is increased as VEGF chaperone (Ozawa et al., 2001); leptin gene

up-regulated by HIF-1 (Ambrosini et al., 2002). In addition, stromal cell-derived factor 1 (CXCL12) is controlled by HIF-1α and boosts VEGF expression and up-regulates migratory inhibitory factor (MIF) (Goel and Mercurio, 2013, Bacher et al., 2003). Likewise, hypoxia up-regulates angiopoietins cell-specific molecules (Ang-1, -2, -3 and -4 family) which up-regulate angiogenesis by binding to Tie receptors (Makrilia et al., 2009), the placenta growth factor (PlGF) expression (Green et al., 2001), and many other polypeptide angiogenic factors, such as pyruvate and lactate which are glycolysis metabolites (Murray and Wilson, 2001).

Reactive oxygen species (ROS) also has a contribution in signaling pathways of many biological cellular functions such as angiogenesis and tumor growth (Xia et al., 2007). It has been established that the endogenous hydrogen peroxide (H_2O_2) enhances VEGF10. It can also induce the angiogenesis cascade steps such as endothelial cell proliferation and migration (Hassan et al., 2014), in addition to microtubules morphogenesis (Shono et al., 1996). Hypoxia produces ROS, this increases the rate of angiogenesis processes (such as tube formation), thus antioxidant agents plays crucial role in modulating the angiogenesis process as their scavenging activity can reduce ROS which in turn influences angiogenesis (Lelkes et al., 1998).

1.9 Antiangiogenesis regulating factors:

The angiogenesis process is modulated by the activity of proangiogenic and antiangiogenic factors. Pathologically, the abnormal vascularization, which is resembled by sprouting or inhibition of the new blood vessels growth, is governed by perturbing the balance taking place among these factors (Tonini et al., 2003). There are many antiangiogenic factors that affect vascularization such as angiostatin, vasostatin, thrombospondins (TSP) (trimeric 450 kDa glycoproteins), interleukins, endostatin, interferons, calreticulin, and metalloproteinases inhibitors which are secreted from the dormant tumors to ensure the stability of the tumor size; at the same time, they have a positive effect on endothelial cell apoptosis (Tonini et al., 2003, Folkman, 2002).

Angiostatin consists of plasminogen fragments, it inhibits extracellular matrix of endothelial cells but on the other hand it opposes the plasminogen up-regulated effect of invasion and migration. The combination of endostatin and angiostatin will lead to strong synergistic antiangiogenic activity (Zhang et al., 2012). Vasostatin is an amino acid domain of calreticulin, it represses proliferation and angiogenesis of endothelial cells (Shu et al., 2014). TSP-1 and the pigment epithelium-derived factor are responsible in controling endothelial cell apoptosis and suppress VEGF expression. They inhibit the basic fibroblast growth factor (bFGF) expression to block angiogenesis (Karahuseyinoglu et al., 2013, Volpert et al., 2002).

Endostatin consists of collagen fragments; it is produced in the walls of the blood vessels. Its function on endothelial cells is to increase apoptosis and down-regulates the

invasion and migration cascade, and this occurs through the inhibitory effect on protein and cyclin D1 mRNA and hyper phosphorylated expression of the retinoblastoma gene (Alahuhta et al., 2015). Interferons alpha and beta (IFN-α and IFN-β) inhibit VEGF expression and they mediate endothelial cell proliferation and suppression of angiogenesis of neuroendocrine tumors (Von Marschall et al., 2003).

1.10 Cell death and apoptosis:

Cell death is a key event in cell biology. Cell death occurs normally via two biological processes; either apoptosis or autophagy which are both forms of programmed cell death. However, cell death might happen also by necrosis which is non-physiological process. Necrosis is an abnormal process caused by external factors as a result of injury or infection. (Kerr et al., 1972, Elmore, 2007). Apoptosis, programmed cell death, identified initially through the morphological changes of dying cells exhibiting specific characteristic patterns which are active membrane blebbing, cytoplasmic shrinkage, chromatin condensation, and, typically, fragmentation into membrane enclosed vesicles known as apoptotic bodies (O'Driscoll et al., 2006). Apoptosis is firmly controlled by complex molecular signaling systems. It plays a key role throughout the cell cycle and development, and it has a direct impact on cell stages like cell ageing, tissue remodeling, and morphogenesis. In this process the cells activate apoptotic genes and kill themselves in a controlled way. Apoptosis causes cells shrinkage during this process to rapidly being digested, thus, there are no leakages of their contents (Raff, 1998). Figure 1.1 demonstrates the morphological changes occurring during the apoptosis process.

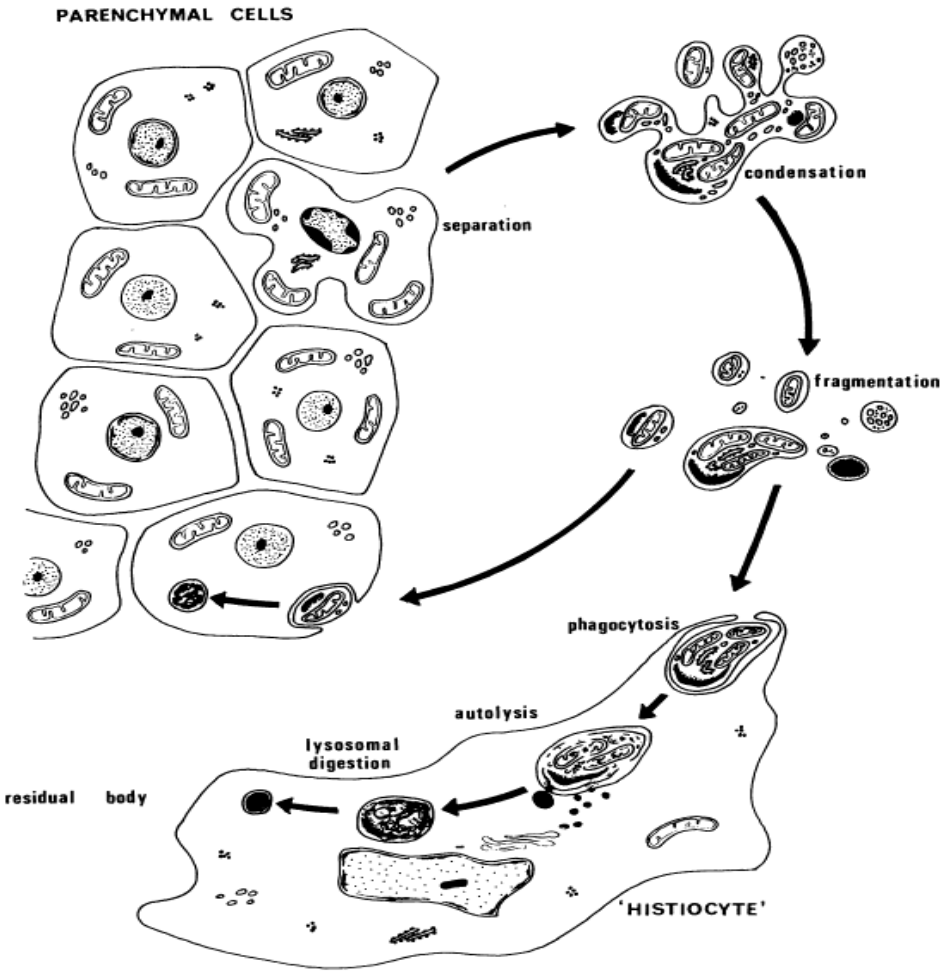

Figure 1.1 Morphological changes occurring during apoptosis (Kerr et al., 1972).

Apoptosis is regulated through intrinsic and extrinsic biochemical pathways. They both lead to activate caspases as the final step (Hengartner, 2000). The intrinsic apoptotic pathway play a crucial role in down-regulation of anti-apoptotic proteins from the bcl-2 family (e.g. bcl-x_L), an increase in mitochondrial membrane permeability and an increased release of cytochrome c into the cytoplasm, which in turn activates caspase-9 and caspase-3 resulting in apoptotic damage (Yon et al., 2005). The extrinsic pathway is initiated by the activation of death receptors that involves the formation of a death-inducing signaling complex (DISC), which contains Fas, a member of the TNF-α superfamily. DISC formation results in the activation of caspase-8, which activates caspase-3 and executing the cell (Medema et al., 1997).

Necrosis is a term that describes uncontrolled process in which death takes place after exposure to an acute injury. The cells start to swell and burst then they spill their content out over the surrounding tissue and that leads to inflammation (Kaku et al., 2016). Table 1.2 illustrates the differences between some of the major features of apoptosis and necrosis. The unregulated rate of apoptosis in the body, either increasing or reducing can cause many ailments, for examples, hematologic diseases (e.g., lymphocytopenia and plastic anemia), apoptosis hyper-activation associated with neurodegenerative diseases (e.g., Alzheimer‘s syndromes and Parkinson’s), and disease characterized with tissue damage (e.g., myocardial infarction). In contrast, reducing apoptosis rate causes cell survival and it is related to autoimmunity diseases (e.g., Systemic lupus erythematosus) and immortal cancer cells (Chamond et al., 1999). The profound understanding to the link between apoptosis and cancer has been realized through studying the kinetics of

tumor growth. These studies have shown the link between cell death and tumor growth. Whereas, the treated tumor with cytotoxic agents showed a high rate of apoptosis occurrence (Kerr et al., 1972).

Table 1.2 Comparison between Apoptosis and necrosis (Ronco et al., 2009).

Feature	**Apoptosis**	**Necrosis**
Exfoliation of cells	Early, as single cells	Late, as sheet of cells
Plasma membrane structure	Blebbing	Lost
Plasma membrane integrity	preserved	Lost
Cell-cell adhesion	Lost early	Preserved
Cell matrix adhesion	Lost early	Lost late
Cytosolic content	Preserved	Released
Chromatin	Condensed	Preserved
Nuclear fragmentation	Characteristic	Absent
Apoptotic bodies	Characteristic	Absent
Phagocytosis	Characteristic	Absent
Cell volume	Decreased	Increased
Inflammation response	Absent	Characteristic

1.11 Signal transduction pathways in cancer:

Cell cycle machinery is regulated by a number of pathways and related proteins, and any disturbance in these regulatory will lead to malignancy (Ghobrial et al., 2005, Kaufmann and Hengartner, 2001). As a kind of adaptation, cancer cells pursue to change the surrounding microenvironment to assist their growth and proliferation. These changes take place when the tumor is exposed to any internal or external stimuli and respond through increasing or decreasing expression of certain proteins to ensure tumor stability, proliferation, invasion, and metastasis (Hanahan and Weinberg, 2000). The signal transduction pathway, which is intracellular biochemical reactions, is mainly responsible for controlling the expression of these certain proteins (Lobbezoo et al., 2003). Each pathway depends on activation of extracellular receptor. Activation of the receptor is interpreted into biological response and activates 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 found to be hyper-activated in cancer cells (Clevers, 2004, Soucek et al., 2008). In contrast, mutations in suppressor genes cause deactivation of some pathways which may act as checkpoints of cells proliferation such as p53 (Lu et al., 2013). In order to treat cancer, these pathways can be targeted mainly with signal transduction modulators (STMs) to suppress tumor growth. The

STMs can control the pathway activity at many levels such as blocking receptors on cell surface, extracellular signals and the transcription factor, deactivation of the binding sites between the transcription factors with the promoters or repress the effects of further downstream genes (Lobbezoo et al., 2003). Many studies are targeting STMs and investigations reached an advanced stage pre-clinically and in clinical trials. Nowadays, there are several approved STMs drugs which are commercially available in the market; Nilotinib, trastuzumab, ponatinib, and imatinib (Zhao et al., 2014).

1.11.1 Wnt /β-catenin signaling pathway:

Wnt signaling pathway plays a key role for development process in normal cells as well as cancer by controlling gene expression, cell adhesion, cell polarity and cell behavior (Cadigan and Nusse, 1997). Wnt signals work through three pathways: Wnt/JNK pathways, Wnt /β-catenin pathway (known as canonical Wnt pathway) and the non-canonical Wnt/Ca+2 (Clevers and Nusse, 2012).

Researchers have discovered mutations in many components of Wnt /β-catenin pathway associated with many types of human cancers such as: melanoma, breast, prostate, and colon cancers (Morin et al., 1997, Verras and Sun, 2006, Chien et al., 2009). In addition, in colon cancer patients, studies revealed that 80% have mutations in APC (tumor suppressor gene) which its function was referred to as a down-regulator of Wnt pathway (Clevers and Nusse, 2012). The Wnt /β-catenin pathway regulates the expression of many of important oncogenes such as: cyclin D1, matrix metalloproteinase, and c-Myc genes which are pivotal in carcinogenesis as well as angiogenesis (Dihlmann and von

Knebel Doeberitz, 2005). So, targeting Wnt pathway can repress the tumor proliferation with aim of downregulation of this pathway (King et al., 2012).

1.11.2 Notch signaling pathway:

Notch cell signaling pathway contributes in various cellular functions such as proliferation, cell fate specification, apoptosis, differentiation, migration, angiogenesis, and adhesion (Takebe et al., 2014). The signaling cascade starts with the ligation of the four isoforms of Notch extracellular receptors (Kojika and Griffin, 2001). A study on T-cell lymphoblastic leukemia patients presented the relation of Notch pathway with cancer, where there was 10% of patients possessing a constitutive activation of Notch 1 receptor (Callahan and Raafat, 2001). Furthermore, according to Yabuuchi et al. (2013), the activation of any Notch isoforms will be well correlated with tumor growth and aggressiveness properties. Hyper-activation of Notch pathway signaling has been noticed in many types of cancer, including breast, lung, melanoma, pancreas, renal, and colon cancers (Capaccione and Pine, 2013, Farnie and Clarke, 2007, Sun et al., 2009).

Many researches showed a strong connection between Wnt and Notch pathways in colon cancer (Wynn et al., 2014). Thus, a combination of Wnt and Notch inhibitor may affect synergistically on colon cancer cells (van Es and Clevers, 2005).

1.11.3 p53 signaling pathway:

In 1980, researchers discovered the relation of cancer with p53 gene mutations as existed widely in all cancer cases. More than 50% of cancer patients have suppressed p53 in tumors. The mutations in p53 gene lead to the activation of other oncogenic pathways, and causes tumors to become more aggressive and resistant to chemotherapy as well as radiation (Muller and Vousden, 2013). The main role of p53 is to induce apoptosis and cell cycle arrest and it has been referred to as 'Guardian of the Genome' due to its decisive effect. p53 protein encodes many type of genes which are involved in apoptosis, angiogenesis, and cell cycle. p53 controls cell death particularly through the two apoptotic pathways (intrinsic and extrinsic). p53 activates death receptor DR-5 and Fas genes which are related to the extrinsic pathway as well as Bak, Bax, and Bid proteins which are related to the mitochondrial pathway (Yamasaki, 2003). According to Lowe et al. (1993), the study was performed on p53 knockout-mice and, as a result, a slowdown in the apoptosis process clearly occurred in addition to drug resistance.

1.11.4 TGF-β signaling pathway:

TGF-β signaling pathway is identified as a double-edged sword, the tumor suppressor and oncogenic properties of this pathway have been described in many studies (Katsuno et al., 2013). As a tumor suppressor, one research have found that TGF-β defect- mice were more susceptive to tumor incidence comparing to normal mice (Tang et al., 1998). In one study on transgenic mice where the TGF-β was hyper-activated, it was shown that these mice were more resistant for mammary tumor formation (Pierce et al., 1995). In another study on *in vitro* cancer cells it was found that the cells tend to secret TGF-β proteins more than normal cells (Roberts et al., 1983). And clinically, TGF-β levels in