4-2016

Anti-Breast cancer effect of Rhus Coriaria extract

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ANTI-BREAST CANCER EFFECT OF *RHUS CORIARIA* EXTRACT

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Rabah Iratni

April 2016

Declaration of Original Work

I, Khawlah Najib Athamneh, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Anti-breast cancer effect of Rhus coriaria extract*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Rabah Iratni, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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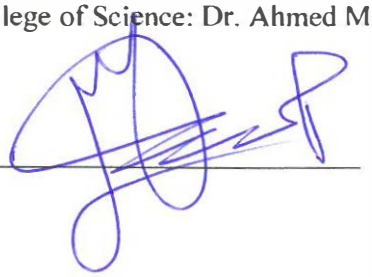
Signature  Date 24/5/2016

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Copy 14 of 14

Abstract

Plants have been shown to be an excellent source of new drugs, including anticancer agents. *Rhus coriaria,* commonly known as sumac, a plant that is known to possess different therapeutic values including anti-oxidant and anti-microbial activities. Here, we investigated the anti-cancer effect of *R. coriaria* on triple negative MDA-MB-231 cell line. We demonstrated that *Rhus coriaria* ethanolic extract (RCE) inhibits the proliferation of MDA-MB-231 cell line in a time- and concentration-dependent manner. RCE induced senescence and cell cycle arrest at G1 phase. Moreover, no proliferative recovery was detected after RCE removal. Annexin V staining and PARP cleavage analysis revealed a minimal induction of apoptosis in MDA-MB-231 cells. Autophagy vacuoles were detected along with autophagy markers. Interestingly, blocking autophagy by 3-methyladenine (3-MA) or chloroquine (CQ) reduced RCE-induced cell death and senescence. Finally, RCE induced DNA damage, an event that was found to precede autophagy. Hence, altogether, these findings make *Rhus coriaria* a promising alternative candidate against breast cancer.

Keywords: *Rhus coriaria*, triple negative breast cancer, apoptosis, senescence, cell cycle, autophagy, DNA damage.

Title and Abstract (in Arabic)

أثر نبات السماق المضاد لسرطان الثدي

الملخص

تستخدم النباتات في صنع الأدوية الجديدة التي تتضمن مضادات السرطان. أحد هذه النباتات هو نبات ال *(Rhus coriaria)* و يعرف بنبات السماق. أشارت الدراسات السابقة على فاعلية نبات السماق كعلاج مضاد للأكسدة ومضاد حيوي. وبالتالي قمنا في هذه الأطروحة بدراسة أثر مستخلص السماق على سرطان الثدي. ولقد توصلنا في هذا الدراسة إلى أن نبات السماق يمنع نمو وتكاثر الخلايا السرطانية (MDA-MB-231) بالاعتماد على الوقت والتركيز. كما أنه أدى إلى إيقاف دورة حياة الخلية (cell cycle arrest) وإلى الشيخوخة (senescence) في الخلايا السرطانية. بالإضافة إلى ذلك فإن نبات السماق منع تكاثر الخلايا السرطانية بعد إزالة السماق بل وأدى إلى موتها حتى بعد إزالة السماق من الخلايا. لقد وجدنا أن بعض الخلايا تموت بعملية الموت الذاتي (apoptosis)، ولكن العملية الرئيسية التي وجدناها لموت الخلايا السرطانية هي الموت الذاتي للخلايا (autophagy). وعندما منعنا عملية الموت الذاتي بمواد كيميائية خاصة-3 (MA أو CQ) قلت نسبة الشيخوخة وموت الخلايا. كما وجدنا أن السماق يؤدي إلى إلحاق الضرر بالحمض النووي (DNA damage) الخاص بالخلايا السرطانية. كل هذه النتائج تجعل نبات السماق بديل واعد كمضاد لسرطان الثدي.

مفاهيم البحث الرئيسية: نبات السماق، الشيخوخة، دورة حياة الخلية،الموت المبرمج للخلايا، الموت الذاتي للخلايا، ضرر الحمض النووي.

Acknowledgements

This thesis could not have been completed without the great support that I have received from people to whom I am greatly indebted.

First and foremost, I offer my deepest gratitude to my supervisor and mentor, Dr. Rabah Iratni, for supporting me throughout my studies with his outstanding guidance, caring and patience. He taught me to pursue knowledge for the sake of it, and to be passionate about my work. I would thank him for spending countless hours in the lab explaining and teaching not only science but also life. He was there whenever I needed his help regardless of the tremendous amount of work that he has. I would thank him for believing in me and allowing me to realize my own potential. He taught me a lot of things yet there are much more to learn from him. One simply could not wish for a better supervisor.

I would also like to express my thanks to Dr. Yusra Al-Dhaheri for her guidance and advices through my studies. I am also indebted to Mr. Hussain El Hasasna and Mr. Noushad Karuventevida for their continuous support and help. Whenever I ask for help they were there to offer more than I asked. Also, Nesreen Al Faresi for her help and guidance when I entered the lab.

A special thanks to Halima Al Samri who without my master journey will not be as joyful. From the day I began graduate school, she has become one of my best friends. She helped me through both good times and bad and she was always there for me. I will always remember the great conversations we had over lunch, and I know that we will break bread together many more times.

I would also like to thank my friend Asma Al Rashedi whom I have always been able to turn to when I needed a listening ear or a wise opinion. She was a great friend and a rock of stability in my life. She made me see the world in a deep and different way and she taught me new things.

I need to thank the people who create such a good atmosphere in the lab and was there for me, Nehla Benhalilou, Aysha Al Meqbali and Nedaa Al Tamimi.

I would also like to acknowledge my committee members: Professor Stefan Nonchev and Dr. Ranjit Vijayan for accepting to judge my thesis work and for being generous with their expertise and precious time.

I also need to thank the biology department and special thanks to Mrs. Raja Al Meskari and Dr. Mohammed Mousa for their continuous help and support whenever I needed anything. And I need to thank Dr. Soleiman Hisaindee from chemistry department for the extract preparation.

Moreover, I would like to thank all my instructors in my undergraduate and graduate studies for all what they taught me throughout the years and for helping me to follow my dream.

I would also like to thank the graduate studies office for putting up with me and answering all my questions. I also need to thank the library deanship for providing me with all the resources that I needed and responding to all my requests.

I must express my very profound gratitude to all my friends for supporting and encouraging me particularly, Aysha Al Neyadi who was always there supporting and listening to me when practicing and helping me with my thesis, Samar Al Muraqab for

being there for me whenever I needed, and Safeya Al Hamoudi for being there through good times and bad cheering me up and standing by my side when times get hard.

Last but not least, no acknowledgments would be complete without giving thanks to my parents for giving me solid roots from which I can grow and for being a constant and active presence in my life. Mom, especially, was a great role model of resilience, strength and character. My sisters and brothers for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis.

Dedication

To my beloved parents Najib and Khadija Athamneh, and to my second father.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

RCE	*Rhus coriaria* extract
HER2	Human Epidermal Growth Factor-2
UV	Ultra Violet
ER	Estrogen Receptors
PR	Progesterone Receptor
TNBC	Triple Negative Breast Cancer
CDK	Cyclin Dependent Kinase
SA-β-Gal	Senescence Associated β-Galactosidase
ROS	Reactive Oxygen Species
PARP	Poly (ADP-ribose) polymerase

Chapter 1: Introduction

1.1 Cancer

Cancer remains one of the leading causes of morbidity and mortality. It is a major public health problem worldwide. An estimated 14.1 million new cancer cases were diagnosed worldwide in 2012. Sadly, 8.2 million deaths from cancer occurred in the same year. Moreover, it is predicted that by 2020 cancer rates could further increase by 50% to reach 15 million new cases[1]. In the United Arab Emirates, 1212 new cancer cases were reported in 2012. Cancer is the second leading cause of death in the Emirate of Abu Dhabi, and accounts for 15% of total deaths as there were around 407 deaths caused by cancer in 2012[2].

1.1.1 Definition and classification

Cancer is defined as a multifactorial disease that is characterized by uncontrolled cellular growth, invasion and spreading of those cells from their primary site to other sites in the body to establish new colonies of cancer cells[3]. Cancers are classified upon their tissue of origin. The most common of them are carcinomas which arise from epithelial cells, while sarcomas are derived from mesoderm cells. Cancers of epithelial cells with glandular organization are called adenocarcinomas[3]. Other classes of cancers include lymphoma and leukaemia are derived from white blood cells and their precursors[4].

1.1.2 Hallmarks of cancer

Cancer cells are distinguished from normal cells by acquiring specific hallmarks. These hallmarks include stimulating their own growth by responding to

their own signals, sustaining proliferative signalling and resisting inhibitory signals that might otherwise stop their growth. In addition, evading apoptosis and enabling unlimited replicative potential, stimulating angiogenesis, by forming blood vessels to supply nutrients to tumors, invading local tissues and metastasize to distant sites. Furthermore, reprogramming of energy metabolism and evading immune system destruction are emerging hallmarks of cancers that need further study[5].

1.1.3 Causes of cancer

While the real cause of cancer is still unknown, many factors have been associated with cancer. These factors include genetic mutation in tumor suppressor genes and oncogenes, life style, chemical agents and viruses[3].

1.2 Breast cancer

Breast cancer represent the most commonly diagnosed cancers in women. There were 1.7 million new cases diagnosed in 2012 worldwide. This represents about 12% of all cancer cases and 25% of all cancers in women[6]. In the United Arab Emirates the leading cause of death related to cancer for the same year was breast cancer as well[2]. Breast cancers are a heterogeneous and diverse group of diseases that comes in several clinical and histological implications. The clinical progression of breast cancer is difficult to predict and its current treatment is therefore not as effective as it should be[7], [8]. Gene expression profiling using microarray analysis assists in creating a working model for a breast cancer molecular taxonomy which has become widely used. Using hierarchical cluster analysis breast cancers can be classified into one of five molecular subtypes: luminal A and luminal B –both are estrogen receptor (ER)-positive- , basal-like (ER)-negative, human epidermal growth factor receptor 2 (HER2) overexpression , and normal breast-like[7],[9]–[11].

1.2.1 Triple negative breast cancer

Triple-negative breast cancers (TNBCs) represent a heterogeneous subtype of breast cancers that are associated with an aggressive clinical conditions and poor prognosis, where targeted therapies are currently limited[12]. The majority of TNBCs belong to the basal-like category. Their clinical and pathologic features overlap with hereditary BRCA1 related breast cancers[13], [14]. TNBCs are characterized by the absence of ER, progesterone receptor (PR) and lack of HER2 expression[15]. Therefore, TNBCs have more aggressive clinical course than other forms of breast cancers, with an increased likelihood of recurrence and death[16].

1.3 Molecular mechanisms targeted in cancer

Several cellular processes and molecular mechanisms are targeted in cancer treatment. These mechanisms include DNA damage, cell cycle, senescence and cell death.

1.3.1 DNA damage

DNA damage is an alteration in DNA which can be caused by different stressors. These stressors include radiations such as UV light and X-rays, chemicals and reactive oxygen species (ROS)[17]. In response to such stresses, cells activate several mechanisms to repair DNA and maintain genomic integrity[18], [19]. If DNA damage was beyond the cell's repair capacity, then cell cycle arrest and/or cell death might occur to eliminate potentially dangerous mutations[20]. One example of DNA damage is double strand breakages that are detected by γH2AX marker. H2AX histone variant becomes rapidly phosphorylated, upon double strand breaks, at serine 139 to form γH2AX that act as a signal for the recruitments of proteins involved in DNA

repair and chromatin remodelling[21],[22]. As the double strand breakages are repaired, dephosphorylation of γH2AX occur and the cell goes into its normal state[23], [24].

1.3.1.1 DNA damage and cancer

Accumulation of DNA damage that is beyond repair capacity, or defect in DNA repair pathways leads to mutations or chromosomal aberrations that affect oncogenes and tumor suppressor genes. As a result, cells could undergo malignant transformation into cancer[25]. In cancer cells, defects in DNA repair pathways enable cancer cells to accumulate genomic alterations that contribute to their aggressive phenotype[26]. Alterations in DNA repair pathways can make some cancer cells depend on less DNA repair pathways for survival. Moreover, there are specific DNA repair pathways that enable cancer cells to survive DNA damage that is induced by chemotherapeutic treatments, making them resistant to such drugs[27].

1.3.2 Cell cycle

Cancer is characterized by uncontrolled proliferation and dysregulated cell cycle control. Cell proliferation involves the reproduction of a cell to form two daughter cells[28]. Cell cycle consists of distinct phases. The major phases are S and M phases. In S phase, DNA is replicated and chromosomes are duplicated. While M phase consists of two events: nuclear division (mitosis) and cell division (cytokinesis). Interphase comprises the end of M phase and the beginning of the next. Most cell cycles includes gap phases in between S and M phases. G1 occurs before S phase and G2 before M phase. Gap phases provide additional time for cells to grow. It also serve as regulatory transitions that controls the progression to the next stage of cell cycle. G1 is an important phase in which cell is committed into continued division or exit

from cell cycle. In the presence of unfavorable growth conditions cells go into prolonged G1 or exit into non-dividing state called G0[29].

1.3.2.1 Control system

Cell cycle is controlled by a regulatory network[30]. The changes in gene expression as a function of cell cycle progression are regulated by specific cyclin dependent kinases (CDKs) activities[31]. CDKs are protein kinase subunits that forms complexes with a regulatory cyclin proteins. Cyclins are synthesized and destroyed at specific times during cell cycle, thus regulating kinase activity. Human cells contain multiple CDKs and cyclins. However, only certain subset of CDK–cyclin complexes are directly involved in driving the cell cycle. They include three interphase CDKs (CDK2, CDK4 and CDK6), a mitotic CDK (CDK1, also known as cell division control protein 2 (CDC2)) and ten cyclins that belong to four different classes (the A, B, D and E cyclins)[32], [33].

1.3.2.1.1 Checkpoints

There are two crucial aspects of cell cycle regulation: DNA structure checkpoints, which arrest the cell cycle in response to DNA damage or incomplete replication, and commitment point in which a cell becomes committed to enter the cell cycle and progress through it independently of signals from the environment[34].

DNA checkpoints sense possible defects during DNA synthesis and chromosome segregation. They respond to internal stresses such as incomplete replication or external stresses such as DNA damaging agents to block cell cycle progression. Cell cycle arrest allows cells to properly repair these defects, thus preventing their transmission to the resulting daughter cells[35].

The major checkpoints are DNA damage checkpoints: at entry into S phase (G1–S checkpoint) and mitosis (G2–M checkpoint), and the spindle checkpoint that controls progression into anaphase[28]. DNA damage checkpoints protect cells from exogenous and endogenous genotoxic agents that induce diverse alterations in the DNA molecule. These alterations are sensed by signalling pathways that ultimately leads to CDK inhibition and cell cycle arrest[36]. If repair is unsuccessful because of the excessive DNA damage or genetic defects in either the checkpoint or the DNA repair machinery, cells may enter senescence or undergo apoptosis[37]. Spindle assembly checkpoint controls the proper segregation of the chromosomes once the genetic material is duplicated. It has a signalling pathway that modulates CDK1 activity and prevent defects in chromosome segregation[38], [39].

1.3.2.2 Cell cycle and cancer

The loss of checkpoint controls in cancer cells make them less sensitive to the normal growth signals that regulate normal cell proliferation. Tumor cells accumulate mutations that result in constitutive growth signalling and defective responses to anti-growth signals that contribute to unscheduled proliferation[32], [33], [40]. Most tumors acquire genomic instability that leads to additional mutations as well as chromosomal instability[37], [38]. These alterations result in proliferative advantages and increased susceptibility to the accumulation of additional genetic alterations that contribute to tumor progression and acquisition of more aggressive phenotypes. Cell cycle defects are mediated, directly or indirectly, by misregulation of CDKs[41].

1.3.3 Senescence

Senescence was first described by Hayflick and Moorhead as they showed that cells have limited ability to proliferate[42]. Mitotically competent cells respond to

various stressors by undergoing cellular senescence as shown in Figure 1[43]. Cells loose the ability to proliferate while remaining alive.[44]. Senescent cells adopt morphological changes that are characterized by flattened shape and enlarged size[43], [45]. Senescent cells exhibit specific molecular markers such as senescence-associated β-galactosidase (SA-β-gal) and senescence-associated heterochromatin foci (SAHF)[46], [47]. SA-β-gal activity is known to be increased in senescent cells. This increase is likely due to an increased lysosomal content of senescent cells, giving rise to an elevated β-galactosidase activity that becomes detectable at pH 6[45], [48]. However, there is no evidence on the actual involvement of this enzyme in senescence pathway[49].

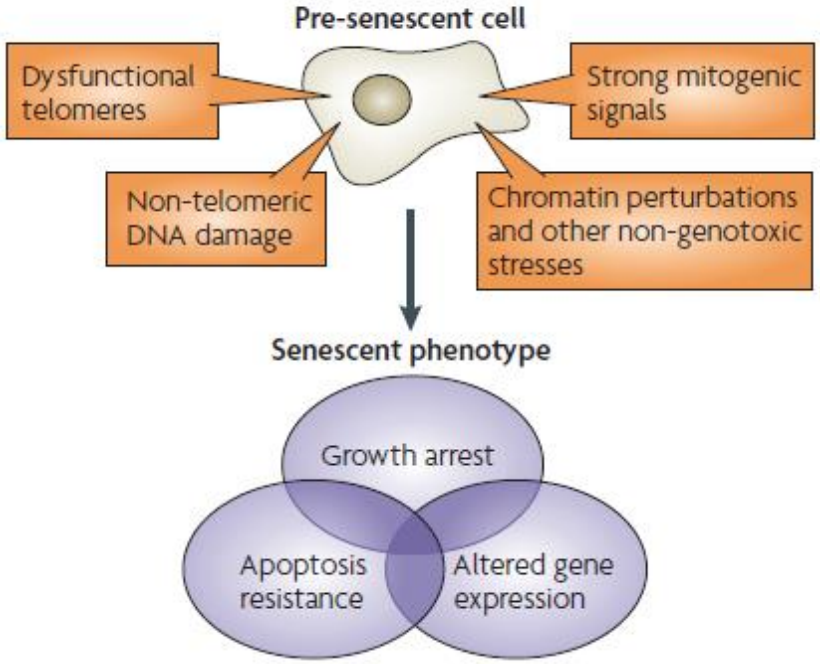


Figure 1: The senescent phenotype induced by multiple stimuli[43].

1.3.3.1 Senescence and cancer

Mouse models of cancer have demonstrated that senescence is associated with pre-malignant stages of neoplastic transformation and has a crucial function in preventing tumor progression. Interestingly, senescent tumor cells are not only growth arrested but can be also cleared by phagocytic cells[50]. Loss or inactivation of tumor suppressor genes are associated with impaired senescence[51]. Therapeutic approaches such as p53-tumor suppressor gene- reactivation, inhibition of c-MYC-oncogene- in tumors or treatment with cyclin-dependent kinase (CDK) inhibitors have proven to be effective by stimulating a senescence response[52]. Therefore, malignant tumors, despite their impaired ability to undergo senescence, can still be forced into senescence if crucial oncogenic pathways are disabled or tumor suppressors are restored. Therefore, senescence-inducing drugs could represent an ideal opportunity to increase the arsenal of anticancer weapons[51].

1.3.4 Programed cell death

Programmed cell death (PCD) is essential for the development and maintenance of multicellular organisms. Two self-destructive processes, autophagy (‘self-eating’) and apoptosis (‘self-killing’), have captivated the imagination of scientists. Thus, apoptosis and autophagy constitute two processes through which damaged or aged cells or organelles are eliminated[53].

1.3.4.1 Apoptosis (programmed cell death I)

Apoptosis is a tightly regulated multi-step pathway responsible for cell death not only during development, but also in adult multicellular organisms to maintain homeostasis. Key characteristics of apoptosis are cellular shrinkage, condensation of the nucleus and DNA fragmentation[54], [55] . Cells that undergo apoptosis initially

become rounded and retracted from neighboring cells which is accompanied by plasma membrane blebbing[56], [57]. A dominant signal of apoptosis is the translocation of phosphatidylserine from the inner to the outer side of the plasma membrane. This ‘eat-me’ indicator functions as a recognition signal for phagocytic cells to engulf apoptotic cells[58]. Apoptosis occur in a controlled manner to minimize damage and disruption to neighboring cells[54]. Apoptosis is orchestrated primarily, but not exclusively, by members of cysteine proteases family known as caspases[59].

1.3.4.1.2 Apoptosis and cancer

To cope with DNA damage, cells have evolved a sophisticated repair system. Failure of this system leads to genomic instability, which triggers apoptosis under normal physiological circumstances. Mutations in key apoptosis signalling proteins and oncogene activation result in evading apoptosis, which ultimately lead to tumor development[58].

1.3.4.2 Autophagy (programmed cell death II)

Autophagy is a highly regulated process that can either be involved in the turnover of long-lived proteins and whole organelles or can specifically target distinct organelles (for example, mitochondria in mitophagy and the endoplasmic reticulum (ER) in reticulophagy), thereby eliminating supernumerary or damaged organelles[60].

Autophagy can act as cellular survival mechanism or cell death. Autophagy can protect cells against death, as a cytoprotection mechanisms, autophagy mediates the removal of protein aggregates that otherwise will lead to cellular dysfunction. On the other hand, autophagy can mediate cellular death. One way by killing, where autophagy destroys large proportions of the cytosol and organelles that would cause

irreversible cellular atrophy with a consequent collapse of vital cellular functions. Indeed, during extensive autophagy, the volume that is occupied by autophagic vacuoles and dense bodies may be roughly equal to, or greater than, that of 'free' cytosol and organelles. Autophagy can also mediate cellular death by triggering apoptosis or necrosis as a primary response to stress stimuli[61], [62].

1.3.4.2.1 Mechanism of autophagy

Autophagy starts with the engulfment of cytoplasmic material by the phagophore inside an intact cell, which sequesters material in double-membraned vesicles named autophagosomes.

Among the initial steps of vesicle nucleation is the activation of Vps34, a class III phosphatidylinositol 3-kinase (PI3K), to generate phosphatidylinositol-3-phosphate (PtdIns3P). Vps34 activation depends on the formation of a multiprotein complex in which beclin-1 is involved. This complex recruits autophagy related genes (Atg) that are essential for autophagosome formation. Atg 5, 7 and 8 complex promotes the recruitment and conversion of the cytosolic microtubule-associated protein light chain 3-I (LC3-I) into LC3-II by cysteine protease Atg4. LC3-II will be incorporated into the autophagosome membrane[53], [63]. A simplified scheme of autophagy mechanism is illustrated in Figure 2[64]. LC3-II also binds to the adaptor protein p62/sequestosome1 (SQSTM 1), which is involved in trafficking proteins to the proteasome and serves to facilitate the autophagic degradation of ubiquitinated protein aggregates[65]. p62/SQSTM 1 is normally degraded during autophagy and accumulates when autophagy is impaired, as has been shown in autophagy-deficient mice[66]. Autophagosomes undergo maturation by fusion with lysosomes to form autolysosomes. In the autolysosomes, the inner membrane as well as the luminal

content of the autophagic vacuoles is degraded by lysosomal enzymes that act optimally within this acidic compartment[67].

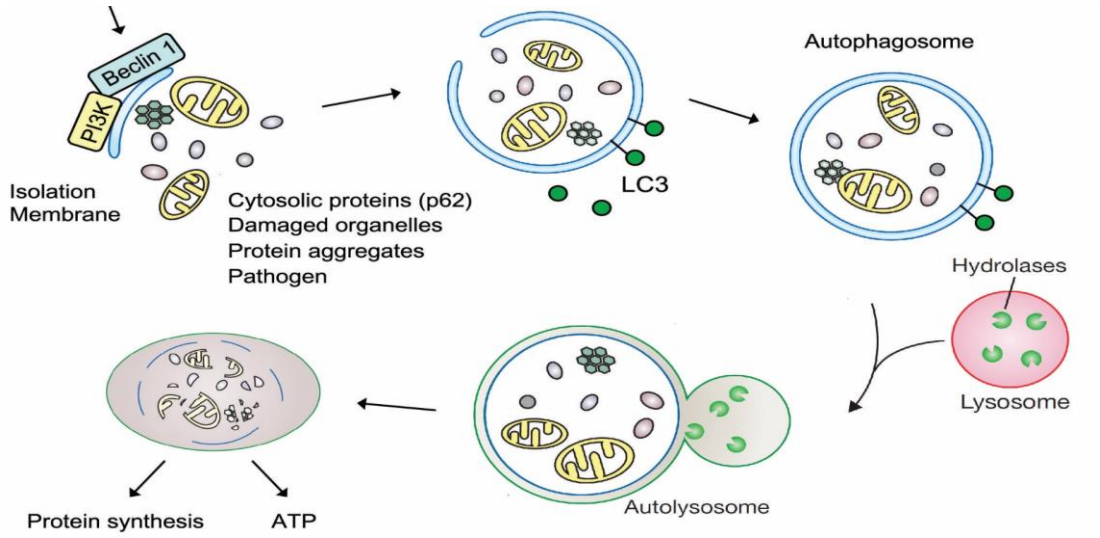


Figure 2: Simplified mechanism of autophagy[64].

1.3.4.2.2 Autophagy and cancer

The role of autophagy in cancer is multifactorial and highly context-dependent. Autophagy is recognized as a survival factor for tumor cells under certain metabolic and therapeutic stresses[63]. However, genetic evidence suggested that autophagy plays a role in tumor suppression[68]. It was proposed that autophagy functions as a tumor suppressor at early stages of tumor development, since the expression of beclin-1, was found to reduce tumorigenesis through induction of autophagy. Conversely, autophagy was found to promote tumor progression at later stages of tumor to survive low-oxygen and low-nutrient conditions. Moreover, it was established that autophagy protects some cancer cells against anti-cancer drugs by blocking the apoptotic pathway. In contrast, other cancer cells undergo autophagic cell death after cancer therapies[69].

1.4 Conventional therapy

Different types of cancer therapy are used for cancer treatment depending on cancer type and stage. These treatments include the following: surgery, radiotherapy, chemotherapy.,etc. Surgery aims to remove localized primary tumors. In radiotherapy, ionizing radiation are used to target tumor cells where they react with water to generate reactive oxygen spices (ROS) that damage the DNA[3]. Chemotherapy aims to target highly proliferative cancerous cells.

1.5 Alternative therapy

Therapies used nowadays to treat breast cancer has definitely improved patients' disease status. However, the side effects that are accompanied with such treatments, sometimes lead to death, beside, aggressive metastasized cancer remains untreatable. Therefore, novel therapeutic options are needed to target aggressive type of breast cancer with less side effects.

1.5.1 Plants

Plants have been used as medicine for many diseases throughout the years. They were shown to be a source of new drugs, including anticancer drugs. There are compelling evidences from epidemiological and experimental studies that highlight the importance of compounds derived from plants "phytochemicals" to reduce the risk of several cancers and inhibit the development of tumors in experimental animals. With advanced knowledge of molecular science and improvement in isolation and purification techniques, many anticancer agents derived from medicinal plants have been identified and developed. More than 25% of drugs used during the last 20 years

are directly derived from plants, while another 25% are chemically altered natural products[70].

Anticancer drugs derived from plants includes taxol isolated from the bark of *Taxus brevifolia* Nutt, vinblastine and vincristine isolated from *Catharanthus roseus*, camptothecin derivatives isolated from the Chinese *Camptotheca acuminata* Decne, and etoposide derived from epipodophyllotoxin are in clinical use. Several other types of promising bioactive compounds of plant origin are currently in clinical trials or preclinical trials or undergoing further investigation[71].

Moreover, recent studies have tested the crude extract of herbal plants to test their anti-cancer effects. For example *Origanum majorana* extract was found to inhibit the viability of triple negative MDA-MB-231 breast cancer cells confirmed by colony growth inhibition, and induced cell cycle arrest in M phase at lower concentration. At higher concentration, apoptosis was induced through the extrinsic apoptotic pathway. Moreover, *Origanum majorana* extract was found to induce DNA damage[72].

In addition to that, several other studies revealed the use of herbal crude extracts to have anti-breast cancer activities. For instance, the aqueous extract of *Fagonia cretica*, used widely as herbal tea, induced cell cycle arrest and apoptosis in MCF-7 and MDA-MB-231 breast cancer cell lines, with activation of DNA damage response[73]. Huanglian extract (*Coptidis rhizoma*), a widely used herb in Chinese medicine, inhibited cellular growth and apoptosis by upregulation of interferon-β and TNF-α in MCF-7 breast cancer cells [74]. *Dillenia suffruticosa* (Griff) ethyl acetate extract caused cytotoxicity to MCF-7 cells and induced apoptosis by modulating several genes which are involved in oxidative stress pathway[75]. An ideal phytochemical is one that possesses anti-tumor properties with minimal or no toxicity and has a defined mechanism of action[76].

1.5.1.1 *Rhus coriaria* Linn

Rhus coriaria Linn, commonly known as sumac, belongs to Anacardiacea family. *R. coriaria* is widely distributed in temperate and subtropical regions[77]. It is considered to be a flowering shrub where the fruit forms clusters of reddish drupes[78]. *R. coriaria* is known to have therapeutic values and dietary qualities; it is used as a medicinal herb, spice and sour drink[79].

1.5.1.1.1 *Rhus coriaria* phytochemicals

According to Abu-Reidah et.al, (2014), 211 phytochemical compounds have been characterized using HPLC–DAD–ESI-MS/MS method. These phytochemicals are organic acids, phenolic acids, phenolic compounds conjugated with malic acid derivatives, flavonoid, isoflavonoid, hydrolysable tannins, anthocyanins, terpenoid and other compounds (such as butein, iridoid and coumarin derivatives) [80].

1.5.1.1.2 *Rhus coriaria* mechanism of action

R. coriaria is considered to be a natural antioxidant; it has the capacity to protect cells and organisms against damage caused by oxidative stress. Previous studies indicated that antioxidant activities of *R. coriaria* extract was due to the presence of phenolic compounds[77], [81]. Several studies linked the accumulation of ROS in the body to different diseases such as atherosclerosis[82], insulin resistance, type II diabetes[83], cardiovascular diseases[84], osteoarthritis[85], hepatocytes toxicity[86] and DNA damage[87]. Interestingly, *R. coriaria* extract was found to have an effect in all the above mentioned diseases. Moreover, it was shown that *R. coriaria* extract induced hypoglycemic activity in type II diabetic rats; as it reduced the postprandial blood glucose (PBG)[88]. In addition, it has antimicrobial activity against gram positive and gram negative bacteria, by causing irreversible damage to

bacteria[89]. A recent study illustrated the cytotoxic and antiangiogenic effects of *R. coriaria* methanolic extract on Y79 retinoblastoma cell line[90].

The aim of this study is to investigate the anti-breast cancer effect of *R. coriaria* on triple negative, highly invasive MDA-MB-231 cell line.

Chapter 2: Materials and Methods

2.1 Cell culture, chemicals and antibodies

Human breast cancer cells MDA-MB-231 and MCF-7 were maintained in DMEM (Hyclone, Cramlington, UK) and T47D in RPMI (Hyclone, Cramlington, UK). All media were complemented with 10% fetal bovine serum (FBS) (Hyclone, Cramlington, UK) and 100 U/ml penicillin/streptomycin (Hyclone, Cramlington, UK). 3-methyadenine (3-MA) and chloroquine (CQ) were purchased from Millipore Analyzer (Millipore, Hayward, CA, USA) and Sigma-Aldrich (Saint-Quentin FAllavier, France), respectively. Antibodies to p62/SQSTMI and cleaved PARP were obtained from Abcam (Abcam, Cambridge, UK). Antibodies to LC3 was obtained from Cell Signaling (USA). Antibodies to γH2AX and Beclin-1 were obtained from Millipore (Millipore, Hayward, CA, USA). Antibody to β-actin were obtained from Santa Cruz Biotechnology, Inc (USA).

2.2 Preparation of the *Rhus coriaria* ethanolic extract (RCE)

Fruits of *Rhus coriaria L.* were collected from a private farm located at 33° 16′ 35.59″ N and 35° 19′ 02.89″ E. The farm is located in Ma'rakeh, Tyre, Lebanon and the approval of the owner was obtained before collecting the fruit or commencing any experiments. This plant is neither endangered nor protected by any laws and it is readily and commercially available in the market. RCE was prepared as previously described[72]. Briefly 10.0 g of the dried fruit were ground to a fine powder using a porcelain mortar and pestle. The powder was then suspended in 100 mL of 70% absolute ethanol and the mixture was kept in the dark for 72 hours at 4 °C in a refrigerator without stirring. After that, the mixture was filtered through

a glass sintered funnel and the filtrate was evaporated to dryness using a rota-vapor at room temperature. The red residue was kept under vacuum for 2–3 hours and its mass was recorded. The residue was stored at −20 °C until further use.

2.3 Measurement of cellular viability

Cells were seeded in triplicate in 96-well plates at a density of 5,000 cells/well. After 24 h of culture, cells were treated with or without various concentrations of *Rhus coriaria* extract for different durations. Cell viability was measured with the Cell Cytotoxicity Assay Kit (Abcam) according to the manufacturer's specifications. The results are representative of an average of 5 independent experiments. Data were presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which is assumed to be 100%.

Cell viability was also measured using the Muse™ Cell Analyzer (Millipore, Hayward, CA, USA) using the Muse Count and Viability Kit (Millipore, Hayward, CA, USA) which differentially stains viable and dead cells based on their permeability to two DNA binding dyes. Briefly, cells were plated onto 12-well plates (50×10^4 cells/well) and allowed to grow for 24 h. The day of treatment cells were counted to estimate the approximate number of cells per well. Following RCE treatment at indicated times, viable cells were counted using Muse™ Cell Analyzer.

2.4 Cell cycle analysis

The cell cycle distribution analysis in control and RCE-treated MDA-MB-231 cells was performed with the Muse™ Cell Analyzer (Millipore, Hayward, CA,

USA) using the Muse™ Cell Cycle Kit (Millipore, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, cells grown onto 6 cm culture dishes were treated with or without various concentrations of RCE. After 24 h or 48 h of treatment, cells were collected by trypsinization, washed in PBS and resuspended in complete media and the Muse cell cycle test reagent was then added to each test tube. Cells were then incubated for 30 min at room temperature in the dark. After staining, the cells were processed for cell cycle analysis. Percentage of cells in G0/G1, S and G2/M phases were determined using the FlowJo software.

2.5 Quantification of apoptosis by Annexin V labelling

Apoptosis was examined using the Annexin V & Dead Cell kit (Millipore, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, MDA-MB-231 cells were treated with or without RCE for 48 h. Detached and adherent cells were collected and incubated with Annexin V and 7-AAD, a dead cell marker, for 20 min at room temperature in the dark. The events for live, early and late apoptotic cells were counted with the Muse™ Cell Analyzer (Millipore, Hayward, CA, USA).

2.6 Hematoxylin-eosin staining of cells

MDA-MB 231 cells (5×10^4) were grown on 2 well labtek chamber slide for 24 h, then treated with and without RCE for 48 h. Cells were then washed twice with PBS and fixed in 10% formalin solution (4% paraformaldehyde) for 5 min at room temperature followed by permeabilization in 70% ethanol. Cells were then washed three times with PBS, stained with hematoxylin for 1 min and washed again before staining with eosin for 30 seconds. For viewing the cells, slides were mounted with

50% glycerol, sealed and observed under Olympus microscope (BX41) fitted with Olympus camera (DP71).

2.7 Senescence-Associated-β-Galactosidase (SA-β-gal) staining

Briefly 10^5 MDA-MB-231 cells were cultured in 6 well plate and treated with and without RCE for 48 h. Treated and control cells were then washed in PBS, and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature. The SA-β-gal staining was performed as previously described.

2.8 Whole cell extract and western blotting analysis

Cells (1.8×10^6) were seeded in 10 cm culture dishes and cultured for 24 h before addition of RCE. After incubation, cells were washed twice with ice-cold PBS, scraped, pelleted and lysed in RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche). After incubation for 30 min on ice, cell lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. Protein concentration of lysates was determined by BCA protein assay kit (Thermo Scientific) and the lysates were adjusted with lysis buffer. Aliquots of 25 μg of total cell lysate were resolved onto 8–15% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Thermo Scientific) and blocked for 1 h at room temperature with 5% non-fat dry milk in TBST (Tris-buffered saline with 0.05% Tween 20). Incubation with specific primary antibodies was performed in blocking buffer overnight at 4 °C. Horseradish peroxidase-conjugated anti-IgG was used as secondary antibody. Immunoreactive bands were detected by ECL chemiluminescent substrate (Thermo Scientific). Where needed, membranes were

stripped in Restore western blot stripping buffer (Thermo Scientific) according to the manufacturer's instructions.

2.9 Statistical analysis

The statistical analysis were done using SPSS version 21. Data were reported as group mean ± SEM. The data were analyzed via t-test, univariate test and one-way ANOVA followed by LSD's *Post-Hoc* multiple comparison test (to compare all groups). Significance for all statistical comparisons was set at $p < 0.05$ using a two-tailed test.

Chapter 3: Results

3.1 The inhibitory effect of *Rhus coriaria* extract on MDA-MB-231, MCF-7 and T47D breast cancer cell lines

We wanted first to test the anti-cancer activity of *Rhus coriaria* extract (RCE) on breast cancer cells. Toward this, we measured the effect of different concentrations of the RCE (0, 50, 100, 200, 400 and 600 µg/mL) on the proliferation of three different breast cancer cell lines (MDA-MB-231, MCF-7 and T47D). Our results indicate that RCE decreased cellular viability in concentration and time-dependent manners (Fig. 3A–C).

By determining the IC_{50} –which is the concentration at which the drug cause 50% inhibition - for each cell line, it appears that T47D and MDA-MB-231 exhibit a greater sensitivity to RCE compared to the MCF-7 cells (Table 1). Then we focused on MDA-MB-231 cells since it belongs to the highly aggressive triple negative breast cancer for further investigation.

IC_{50} (µg/mL)			
	24 h	**48 h**	**72h**
MDA-MB-231	**437**	**305**	**283**
T47D	**374**	**261**	**229**
MCF-7	**ND**	**510**	**433**

Table 1: IC_{50} values for each cell line at the indicated time of treatment

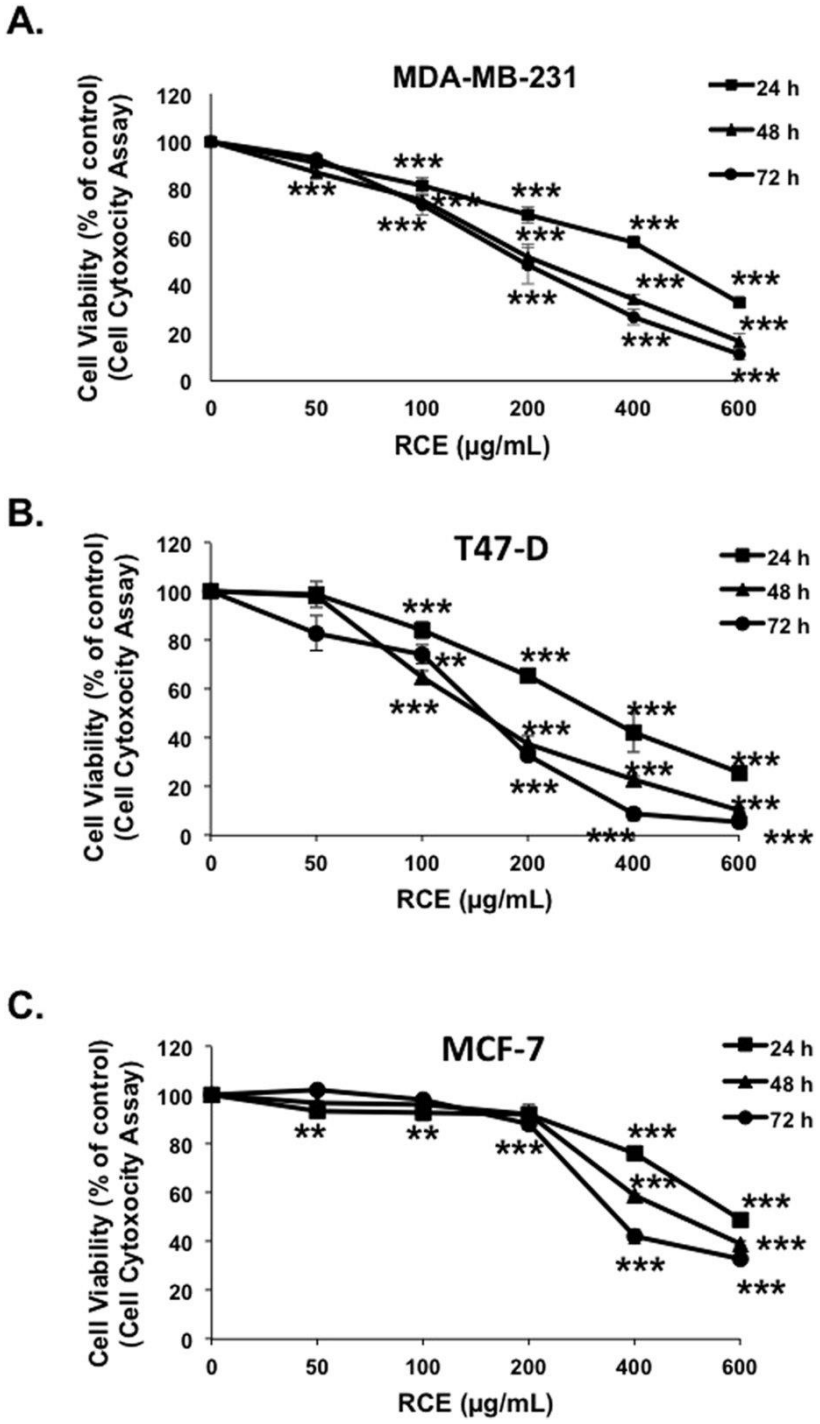
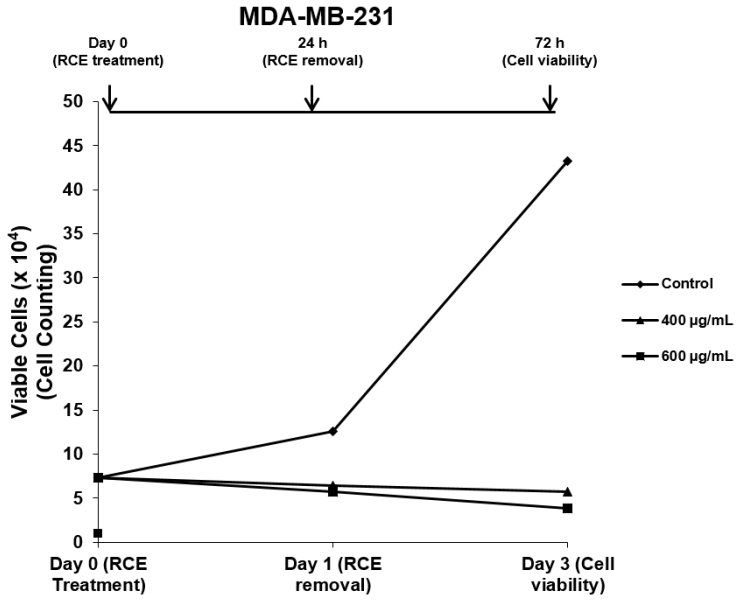


Figure 3: *Rhus coriaria* extract (RCE) inhibited cellular viability of (A)MDA-MB-231, (B) T47-D and (C) MCF-7 breast cancer cell lines treated with or without the indicated concentrations of RCE for 24h, 48h and 72h. Data represent the mean of five independent experiments carried out in triplicate. Statistical analysis for cell viability data was performed using one-way ANOVA followed by LSD *Post-Hoc* test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

3.2 Inhibition of proliferation recovery after removal of *Rhus coriaria* extract

Next, we examined whether or not, RCE can suppress the potential of breast cancer cells to recover proliferative capability. Cells were first treated with the indicated concentration of RCE for 24 h, and then washed with PBS and placed in fresh complete media in the absence of RCE, and allowed to grow for another 48 h before assessing cell viability by cell counting. Figure 4, indicates that MDA-MB-231 cells failed to recover proliferative capability as the number of viable cells kept reducing even after RCE removal. Thus, our result indicates that RCE exerts an irreversible anti-proliferative effect on breast cancer cells.



Total Viable Cells (x 10^4)			
	Day 0 (RCE treatment)	Day 1 (RCE removal)	Day 3 (Cell viability)
Control	7.30 ± 0.39	12.60 ± 0.37	41 ± 1.3
400 µg/mL		6.45 ± 0.27	5.76 ± 0.6
600 µg/mL		5.74 ± 0.22	3.8 ± 0.40

Figure 4: Inhibition of cellular viability recovery after RCE removal. MDA-MB-231 cells were exposed to RCE for 24 h, then, cells were washed and allowed to grow for another 48 h in fresh complete media. Cell viability was monitored using the Muse Cell Analyzer. Data represent the mean ± SEM of three independent experiments.

3.3 Induction of G1 arrest in *Rhus coriaria* treated cells

To investigate the mechanism(s) underlying the inhibitory activity of RCE on breast cancer cells, we examined its effect on cell cycle progression. MDA-MB-231 cells were treated with indicated concentrations of RCE for 24 h and 48 h and were subjected to cell cycle analysis. Treating cells with RCE caused a significant inhibition of cell cycle progression in MDA-MB-231 cells at 24 and 48 h leading to an increase in the G1 population.

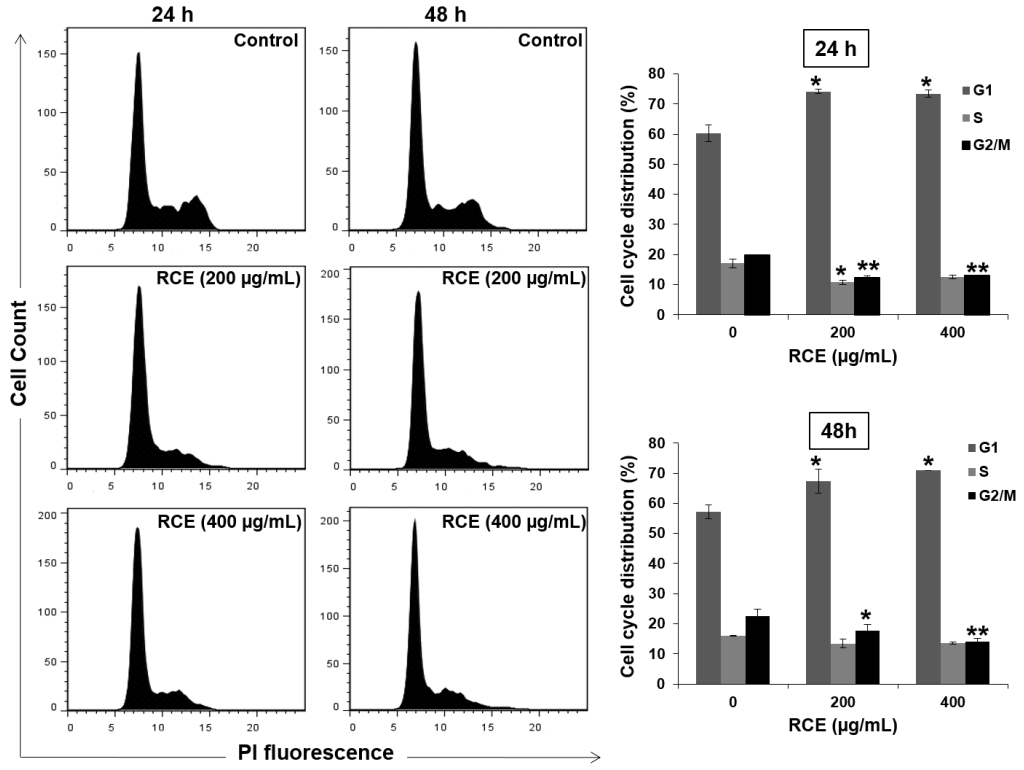


Figure 5: Induction of cell cycle arrest at G1 in MDA-MB-231 cells. Cell cycle distribution analysis of RCE-induced G1 cell-cycle block. MDA-MB 231 cells were first treated with RCE at the indicated concentrations for 24 h and 48 h, and then analyzed with Muse Cell Analyzer.

3.4 Induction of senescence in *Rhus coriaria* treated cells

The induction of cell cycle arrest at G1 phase prompted us to examine if the arrested cells did indeed undergo senescence. Our results show that senescence was detected after treating the cells for 48 h. Almost 21% of the cells that expressed SA-β-galctosidase were detected at 200 µg/mL of RCE. After 96 h of treatment senescent cells nearly doubled at the same concentration. Taken together, these data suggest that induction of senescence might contribute to the inhibitory effect of RCE on the proliferation of MDA-MB-231 cells (fig. 6).

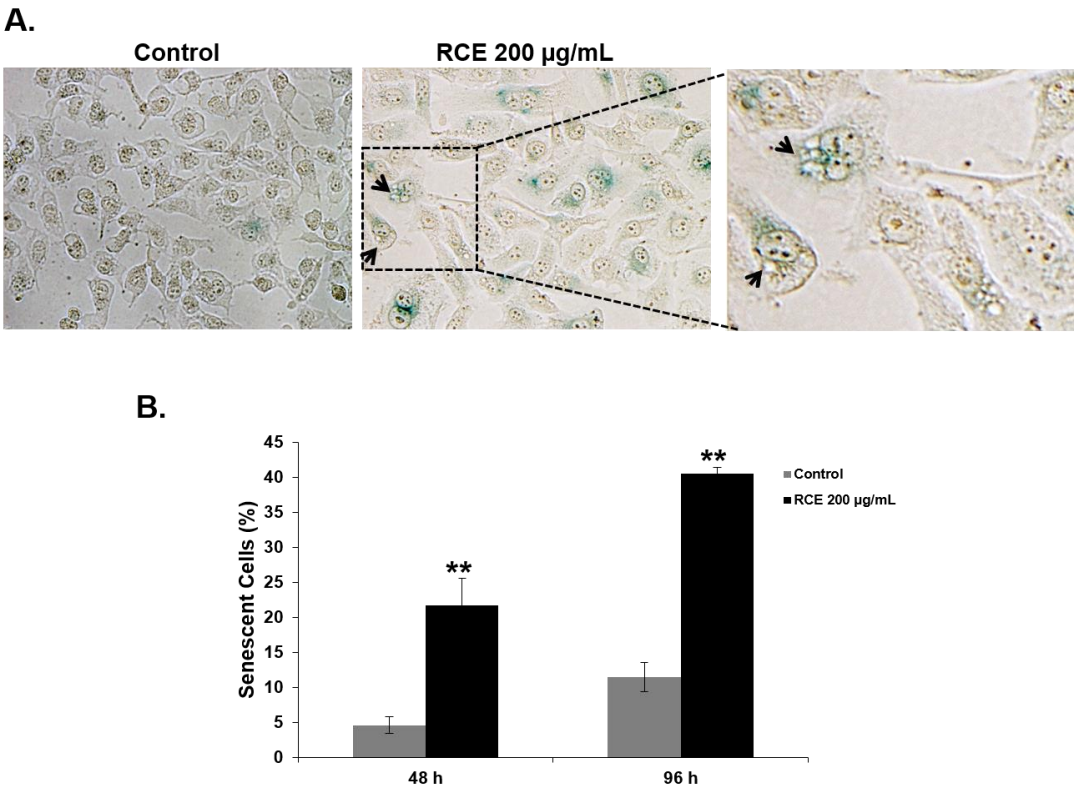


Figure 6: Induction of senescence in MDA-MB-231 cells. MDA-MB 231 cells were incubated with RCE (200 µg/mL) for 48 and 96 hours and stained for SA-β-Galactosidase activity to detect senescence. Data are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

3.5 Minimal induction of apoptosis in MDA-MB-231 treated with *Rhus coriaria* extract

Since RCE decreased cellular viability in MDA-MB-231 cell, we decided to investigate the mechanism by which RCE decreased cellular viability. First we checked whether or not the decreased viability is associated with apoptosis. Therefore, we stained for Annexin V to determine the percentage of apoptotic cells induced by RCE after 48 h treatment. Exposure to RCE did not lead to a significant change in the early stage apoptotic population (Annexin V+/7-AAD−). An increase but still minimal in the late stage apoptotic/necrotic cells (Annexin V+/PI+) (fig.7 AB) was observed at highest RCE concentration, suggesting minimal apoptotic cell death induced by RCE in MDA-MB-231 cells. Apoptosis was further assessed by Poly (ADP-ribose) polymerase (PARP) cleavage. PARP is a nuclear enzyme involved in DNA repair, and it is a well-known substrate for caspase-3 cleavage during apoptosis[91]. Cells treated with etoposide (50 μM) for 24 h, a condition that was reported to induce apoptosis, was used as positive control. Despite the high level of concentration- and time-dependent cell death observed by cell toxicity and cell counting assay during the first 72 h of RCE treatment, very little PARP cleavage, was observed in RCE-treated MDA-MB-231 cells (fig. 7C). Altogether, Annexin V staining and PARP cleavage data support the conclusion of minimal induction of apoptosis by RCE in MDA-MB-231 cells.

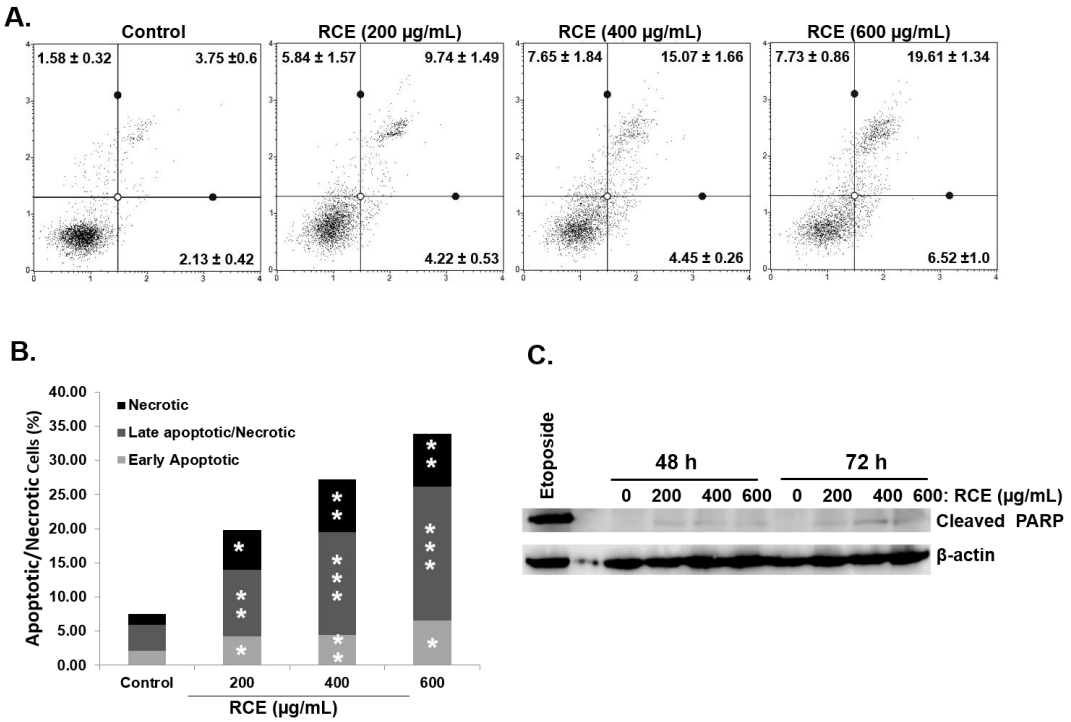


Figure 7: Minimal induction of apoptosis in the MDA-MB-231 cells. **(A-B)** Annexin V binding was carried out using Annexin V & Dead Cell kit (Millipore). Cells were treated with or without increasing concentrations of RCE for 48 h. Detached and adherent cells were collected and stained with Annexin V and 7-AAD and then the events for early and late apoptotic cells were counted by Muse Cell Analyzer. Data represent the mean ± SEM of at least 3 independent experiments. Statistical analysis was performed using ANOVA followed by LSD *Post-Hoc* test to determine the significance (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$). (C) Western blot analysis of PARP cleavage in MDA-MB-231. Cells were treated with increasing concentrations of RCE (200, 400 and 600 µg/mL) for 48 h and 72 h. Exposure of cell to etoposide (50 µM) for 24 h was used as a positive control for apoptosis.

3.6 Induction of autophagy in MDA-MB-231 treated with *Rhus coriaria* extract

Eosin/hematoxylin staining of MDA-MB-231 cells treated with RCE revealed massive cytoplasmic vacuolation that might indicate induction of autophagy (Fig. 8A). In order to determine whether indeed this vacuolation resulted from activation of autophagy. And to further confirm autophagy induction in RCE-treated MDA-MB-231 cells, LC3II accumulation was analyzed by Western blotting

in MDA-MB-231 treated with various concentrations of RCE. RCE induced a concentration-dependent accumulation of the LC3-II (fig. 8B). The expression of p62 (SQSTM1) was also evaluated. There was a concentration-dependent decrease in p62 (SQSTM1) (fig. 8B). Hence, the conversion of LC3I/II along with the downregulation of p62 (SQSTM1) confirm the formation of autophagosome in RCE-treated MDA-MB-231 cells. Next we assessed the expression of beclin-1. Western blotting data showed that the level of Beclin-1 also increased in concentration-dependent manner. Taken together, Western blotting results along with eosin/hematoxylin staining, confirms the activation of autophagy in breast cancer cells in response to RCE treatment.

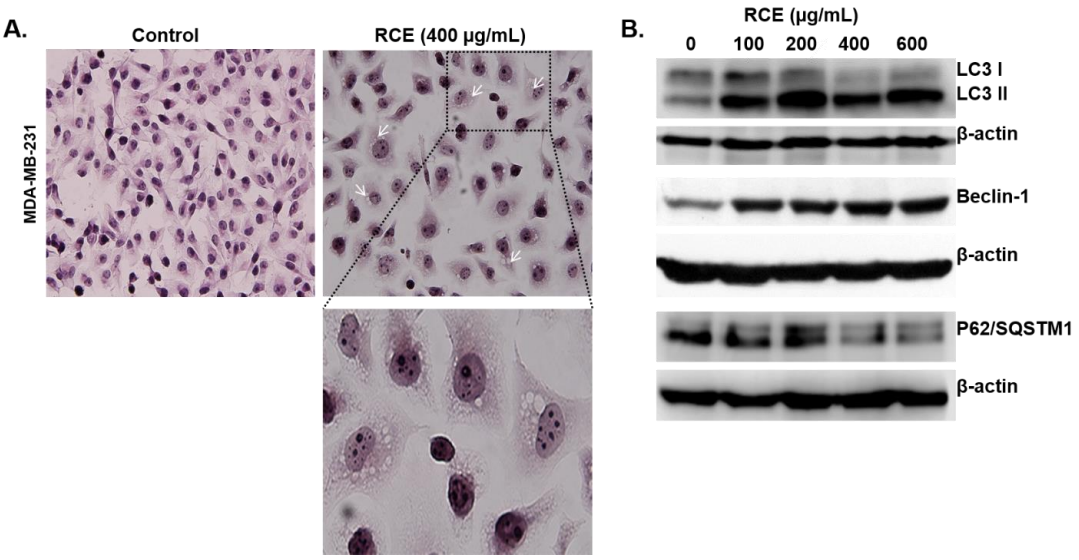


Figure 8: Induction of autophagy in RCE treated MDA-MB-231 cells. (A) MDA-MB-231 cells were treated for 48 h with RCE (400 µg/mL) and then stained with eosin-hematoxylin. (B) Cells were treated with or without increasing concentration of RCE for 48 h, then whole cell proteins were extracted and subjected to Western blot analysis for LC3II, 62(SQSTM1), beclin1 and β-actin (loading control) proteins.

3.7 Autophagy blockage reduces cell death and senescence in *Rhus coriaria*-treated cells

The observation that RCE induces robust cell death in MDA-MB-231 cells and, that induction of apoptosis is minimal raised the question of whether autophagy is responsible for the cytotoxicity activity of RCE through activation of type II programmed cell death and therefore its blockade by autophagy inhibitors might render cells less susceptible to RCE treatment. We used two widely used autophagy inhibitors, 3-methyladenine (3-MA), phosphatidylinositol 3-phosphate kinase (PI3K) inhibitor, which blocks autophagosome formation[69] and chloroquine (CQ), inhibitor of lysosomal acidification, which blocks the fusion between autophagosomes and lysosomes[53]. Results showed that autophagy was markedly inhibited by 3-MA, evident by decreased conversion of LC3-I to LC3-II (fig.9A). However, when cells were pre-treated with CQ, LC3-II protein accumulated to some extent (fig.9A). Next we tested the effect of these inhibitors on the cellular viability of MDA-MB-231 cells treated with RCE. We found that cell viability was markedly improved in the presence of 3-MA or CQ when compared with RCE alone, suggesting that RCE-induced cell death is significantly dependent on autophagy induction (fig. 9B). Furthermore, inhibition of autophagy neither increased nor reduced the level of cleaved PARP, suggesting that RCE-mediated apoptosis and autophagy in MDA-MB-231 might occur independently from each other (fig. 9B). Because blockade of autophagy improved cell viability, and because SA-β-galactosidase was also detected in autophagic cells, we asked the question whether blocking autophagy can also affect senescence in RCE-treated cells. Figure 9D shows that the number of senescent cells in wells containing both CQ and RCE is significantly lower than that in well treated with RCE alone. These results suggest

that autophagy and senescence are linked events and that induction of senescence is at least partly dependent upon the activation of autophagy.

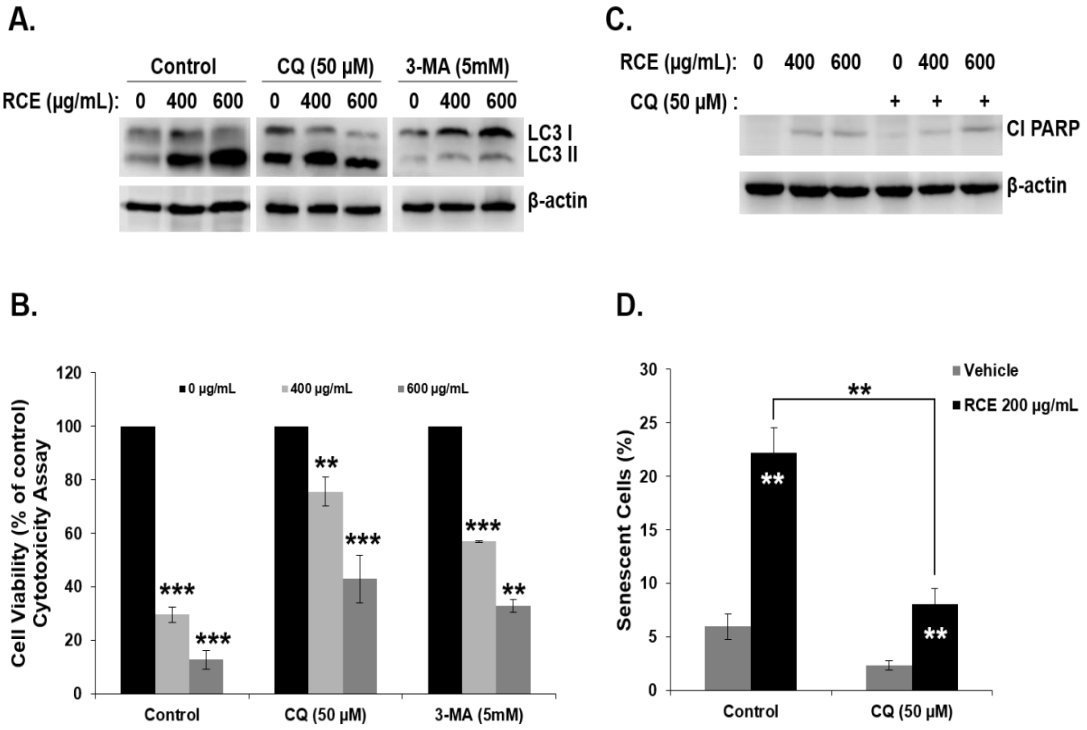


Figure 9: Effects of autophagy inhibitors on cell death, apoptosis and senescence. (A) Analysis of LC3-II accumulation in MDA-MB-231 cells. Cells were pre-treated with or without 3-MA (5 mM) or CQ (50 µM) for 1 h and then RCE was added at the indicated concentrations for 48 h. Proteins were extracted and LC3-II accumulation was determined by western blot. (B) Inhibition of autophagy reduces cell death induced by RCE. MDA-MB-231 cells were pre-treated as described above and treated for 48 h with 400 or 600 µg/mL RCE. Data are representative of three independent experiments. Statistical analysis of cell viability on control or treated cells was performed using one-way ANOVA followed by LSD *Post-Hoc* test to determine significance ($*p < 0.05$, $**p < 0.005$, $***p < 0.001$). (C) Western blot of cleaved PARP in cells pre-treated with and without autophagy inhibitors. (D) Effect of autophagy blockade on RCE-induced senescence. Cells were treated as described in A and stained for SA-β-Gal activity to detect senescence. Data are representative of three independent experiments. Statistical analysis of senescent cells count on control or treated cells was performed using one-way ANOVA and univariate test to determine significance ($**p < 0.01$).

3.8 *Rhus coriaria* induces DNA damage as an early event that precedes autophagy

Next, we sought to investigate whether RCE induces DNA damage in MDA-MB-231 cells. Western blotting analysis revealed a concentration-dependent increase in the levels of phosphorylated H2AX (γH2AX) (fig. 10A), indicating an accumulation of double strand breaks in treated cells. In order to assess whether DNA damage is an early event, a time-course measurement of γH2AX in cells treated with 400 μg/mL RCE was carried out. We found that activation of γH2AX occurred as early as 6 h post-treatment (fig. 10B). To further test whether DNA damage precedes autophagy, MDA-MB-231 cells were first incubated for 1 h with the autophagy inhibitor CQ and then treated with the indicated concentration of RCE. Figure 10C, shows that inhibition of autophagy did not prevent DNA damage in treated cells. Taken together, these results indicate that DNA damage is an earlier event in RCE-treated cells. This damage might then serve as a trigger for downstream responses. Based on these findings, we hypothesize that DNA damage is an early response to RCE that might contribute to the induction of autophagy in MDA-MB-231 cells.

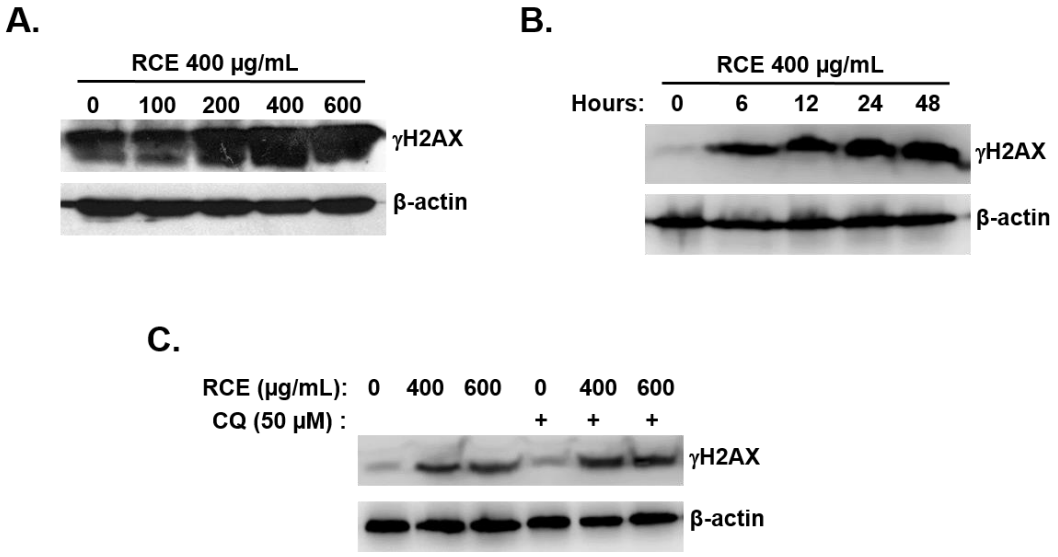


Figure 10: Accumulation of DNA damage in RCE treated MDA-MB-231. (A) Concentration-dependent accumulation of γH2AX, in RCE-treated cells. MDA-MB-231 cells were treated with and without increasing concentrations of RCE for 48 h and DNA damage was analyzed, by determining the level of γH2AX accumulation using anti- phospho-H2AX (ser 139) antibody. (B) Time-course measurement of DNA damage in treated MDA-MB-231 cells. Cells were treated with 400 µg/mL RCE and DNA damage was examined, at different time-point (6, 12, 24 and 48 h). (C) Effect of autophagy inhibitor on the accumulation of DNA damage. Cells were pre-treated with CQ (50 µM) for 1 h before adding RCE (400 and 600 µg/mL) for 48 h. Cells were then harvested and γH2AX level was determined by western blot.

Chapter 4: Discussion

Accumulating evidences demonstrated that several natural compounds derived from plants were found to play a positive role in cancer prevention and treatment through modulating autophagy, which became a promising target for developing potential therapeutic drugs[92]. In our study we investigated the anticancer activity of *Rhus coriaria* on human breast cancer cells. Our work demonstrated that *Rhus coriaria* ethanolic extract (RCE) decreased the cellular viability of three breast cancer cell lines (MDA-MB-231, T47D and MCF-7) in a time- and a concentration-dependent manner. Moreover, it induced an irreversible effect on the survival of the MDA-MB-231 cell line after the removal of RCE since cells were unable to recover their proliferation. RCE induced irreversible cell cycle arrest at G1 phase and senescence detected by the elevated expression of SA-β-Gal. Furthermore, we found that RCE induced autophagy which was the main mechanism of cell death. Induction of DNA damage was also detected by increased expression of γH2AX.

Inhibition of cellular viability by RCE was observed on three breast cancer cell lines although they obtain different characteristics. MDA-MB-231 cell line is originated from adenocarcinoma and the state of its estrogen and progesterone receptors are negative and its p53 gene is mutated. T47D is derived from ductal carcinoma and the state of its estrogen and progesterone receptors are positive and its p53 gene is mutated as well. MCF-7 is originated from adenocarcinoma and the state of its estrogen receptor is positive while the progesterone receptor is negative and its p53 gene is wild type[93]. However we focused on MDA-MB-231 cell line since it belongs to the TNBCs which are characterized by being a highly aggressive form of breast cancer with poor survival rate[94].

Treatment with chemotherapy is not always toxic to all cancer cells; some of the cells survive the treatment, recover and resume their proliferation[95]. Therefore, it is challenging to find drugs that induce irreversible inhibition to uncontrolled cellular growth. Interestingly, our work demonstrated that RCE was able to suppress the capability of MDA-MB-231 cells to recover its proliferative potential at concentrations of 400 and 600 µg/mL, which were the concentrations at which cell death was detected.

It has been proposed that cellular senescence refers to permanent cell cycle arrest in G1 phase in response to different stressors[43] as cells lose the ability to proliferate[44]. In line with the previously published results, our results showed that RCE induced cell cycle arrest at G1 phase and cellular senescence marked by increased expression of SA-β-Gal activity after 48 h of treatment.

Some tumors were found to undergo both autophagic cell death and apoptosis in response to cancer therapy[69]. Apoptosis and autophagy are not always separate events, there might be a crosstalk between them[53]. Here, we showed that RCE prompted minimal apoptosis while the main cellular death mechanism was autophagy. However, the conditions under which autophagy can function as a primary cell death mechanism remain to be defined. As discussed earlier, p62/SQSTM 1 is normally degraded during autophagy and accumulates when autophagy is impaired, as has been shown in autophagy-deficient mice[66]. This, in agreement with our results, which illustrated that P62/SQSTM1 was decreased with increasing concentrations of RCE. Several studies revealed that when autophagy is inhibited, apoptosis is promoted in cancer cells[53]. Unlike these studies, our data showed that when autophagy was inhibited by CQ, there was no induction of apoptosis, suggesting that these two pathways occurred independently.

In cellular context, autophagy has dual function in cellular survival and death. Autophagy promote cellular survival as a protective mechanism due to cellular stressors, however extensive autophagy can lead to cell death[96]. Recently, DNA damage has been shown to induce autophagy, but the exact mechanisms by which DNA damage triggers autophagy are still unclear[97]. As we observed in our results, γH2AX was induced in a dose-dependent manner upon treating the cells with RCE and the inhibition of autophagy did not cause any change in γH2AX expression suggesting that DNA damage is a prerequisite for autophagy. In addition to that DNA damage was also shown to cause cells to undergo senescence[98]. Moreover, it was shown that stress-induced senescence occur in tumor cells *in vitro* and *in vivo* through the exposure of cells to cytotoxic agents that are found to cause DNA damage[44]. Here we demonstrated that cellular senescence was detected after 48 h while DNA damage was detected as early as 6 h suggesting that DNA damage is an early step that precedes cellular senescence. We propose that this damage might serve as a trigger for downstream responses leading to autophagy, senescence and cell death.

It has been shown that increased autophagic activity was associated with senescence in different models, suggesting that autophagy might be an integrated part of senescence program[99]. A positive correlation between autophagy and senescence was also observed where the inhibition of autophagy decreased the number of senescent cells[100]. Moreover, it was shown that inhibition of autophagy by 3-MA decreased the number of SA-β-Gal- positive cells[101]. Here, we showed that low concentration of RCE (200 µg/mL) caused autophagy in association with cellular senescence and when autophagy was inhibited by CQ there was a decrease in senescent cells.

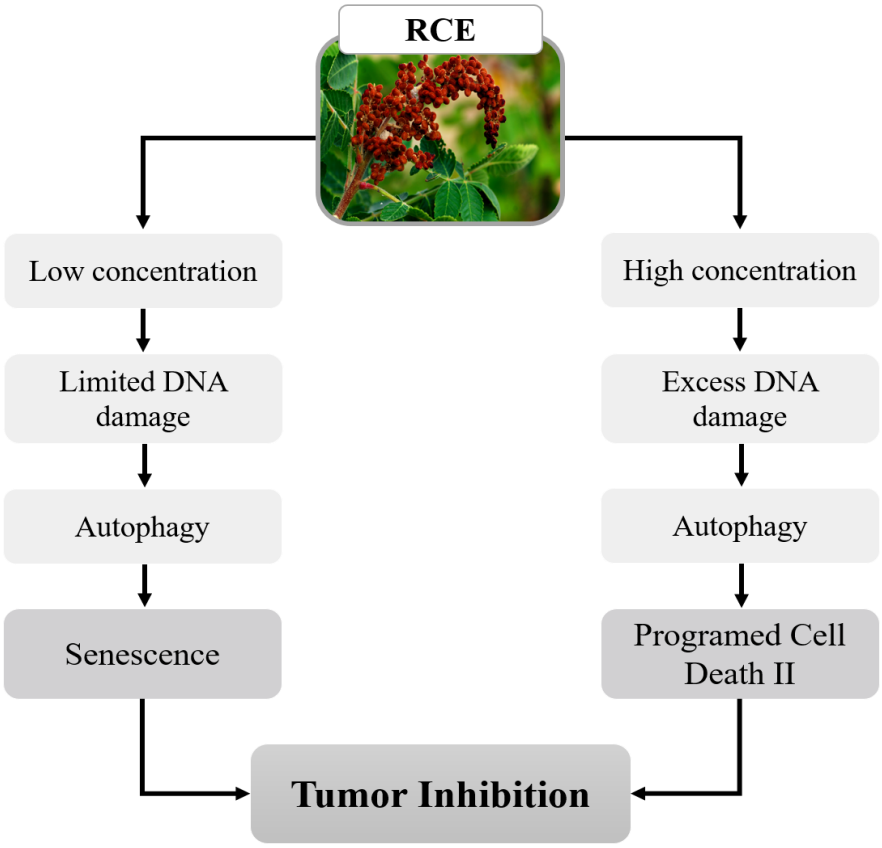


Figure 11: Hypothetical model demonstrating the differential effect of *Rhus coriaria* Extract.

In summary, our data are consistent with a model shown in Figure 11, in which treatment with RCE induce autophagy in MDA-MB-231 TNBC cell line. The magnitude of damage, which depends upon the concentration of RCE, determines the response of the cells. We propose that at lower concentration of RCE (100 and 200 µg/mL) there was a limited induction of γH2AX, where cells respond by triggering autophagy as a survival mechanism followed by cellular senescence. On the other hand, higher concentrations of RCE (400 and 600 µg/mL) caused excessive amounts of DNA damage that was beyond the capacity of the cell to repair, thus results in increased autophagy which ultimately leads to cell death.

Chapter 5: Conclusion

In conclusion, our study demonstrated, for the first time, the potential role of *Rhus coriaria*, as an anti-breast cancer agent *in vitro*. This study provides preliminary data that proposes *Rhus coriaria* as a valuable source of potentially new natural anti-breast cancer compound(s) that act by triggering senescence and autophagic cell death. Further exploration of this plant is urged in order to identify the bioactive phytochemical(s) conferring its anti-breast cancer activity.

Bibliography

[1] "WHO | Cancer," *WHO*. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs297/en/. [Accessed: 03-Oct-2015].

[2] "Cancer Facts & Statistics." [Online]. Available: http://www.haad.ae/simplycheck/tabid/140/Default.aspx. [Accessed: 16-Feb-2016].

[3] L. Pecorino, *Molecular Biology of Cancer: Mechanisms, Targets, and Therapeutics*. OUP Oxford, 2012.

[4] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *Molecular Biology of the Cell*. Taylor & Francis Group, 2007.

[5] D. Hanahan and R. A. Weinberg, "Hallmarks of Cancer: The Next Generation," *Cell*, vol. 144, no. 5, pp. 646–674, Mar. 2011.

[6] "Breast cancer statistics | World Cancer Research Fund International." [Online]. Available: http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/breast-cancer-statistics. [Accessed: 03-Oct-2015].

[7] F. Bertucci and D. Birnbaum, "Reasons for breast cancer heterogeneity," *J. Biol.*, vol. 7, no. 2, p. 6, 2008.

[8] B. Weigelt, A. Mackay, R. A'hern, R. Natrajan, D. S. P. Tan, M. Dowsett, A. Ashworth, and J. S. Reis-Filho, "Breast cancer molecular profiling with single sample predictors: a retrospective analysis," *Lancet Oncol.*, vol. 11, no. 4, pp. 339–349, Apr. 2010.

[9] C. M. Perou, T. Sørlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, Ø. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lønning, A.-L. Børresen-Dale, P. O. Brown, and D. Botstein, "Molecular portraits of human breast tumours," *Nature*, vol. 406, no. 6797, pp. 747–752, Aug. 2000.

[10] T. Sørlie, C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P. E. Lønning, and A. L. Børresen-Dale, "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 19, pp. 10869–10874, Sep. 2001.

[11] T. Sorlie, R. Tibshirani, J. Parker, T. Hastie, J. S. Marron, A. Nobel, S. Deng, H. Johnsen, R. Pesich, S. Geisler, J. Demeter, C. M. Perou, P. E. Lønning, P. O. Brown, A.-L. Børresen-Dale, and D. Botstein, "Repeated observation of

breast tumor subtypes in independent gene expression data sets," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 14, pp. 8418–8423, Jul. 2003.

[12] A. Grigoriadis, A. Mackay, E. Noel, P. J. Wu, R. Natrajan, J. Frankum, J. S. Reis-Filho, and A. Tutt, "Molecular characterisation of cell line models for triple-negative breast cancers," *BMC Genomics*, vol. 13, p. 619, 2012.

[13] B. S. Yadav, P. Chanana, and S. Jhamb, "Biomarkers in triple negative breast cancer: A review," *World J. Clin. Oncol.*, vol. 6, no. 6, pp. 252–263, Dec. 2015.

[14] S. R. Lakhani, J. S. Reis-Filho, L. Fulford, F. Penault-Llorca, M. van der Vijver, S. Parry, T. Bishop, J. Benitez, C. Rivas, Y.-J. Bignon, J. Chang-Claude, U. Hamann, C. J. Cornelisse, P. Devilee, M. W. Beckmann, C. Nestle-Krämling, P. A. Daly, N. Haites, J. Varley, F. Lalloo, G. Evans, C. Maugard, H. Meijers-Heijboer, J. G. M. Klijn, E. Olah, B. A. Gusterson, S. Pilotti, P. Radice, S. Scherneck, H. Sobol, J. Jacquemier, T. Wagner, J. Peto, M. R. Stratton, L. McGuffog, D. F. Easton, and Breast Cancer Linkage Consortium, "Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype," *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.*, vol. 11, no. 14, pp. 5175–5180, Jul. 2005.

[15] F. Podo, L. M. C. Buydens, H. Degani, R. Hilhorst, E. Klipp, I. S. Gribbestad, S. Van Huffel, H. W. M. van Laarhoven, J. Luts, D. Monleon, G. J. Postma, N. Schneiderhan-Marra, F. Santoro, H. Wouters, H. G. Russnes, T. Sørlie, E. Tagliabue, A.-L. Børresen-Dale, and FEMME Consortium, "Triple-negative breast cancer: present challenges and new perspectives," *Mol. Oncol.*, vol. 4, no. 3, pp. 209–229, Jun. 2010.

[16] R. Dent, M. Trudeau, K. I. Pritchard, W. M. Hanna, H. K. Kahn, C. A. Sawka, L. A. Lickley, E. Rawlinson, P. Sun, and S. A. Narod, "Triple-negative breast cancer: clinical features and patterns of recurrence," *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.*, vol. 13, no. 15 Pt 1, pp. 4429–4434, Aug. 2007.

[17] V. Pagès and R. P. P. Fuchs, "How DNA lesions are turned into mutations within cells?," *Oncogene*, vol. 21, no. 58, pp. 8957–8966, Dec. 2002.

[18] S. P. Jackson and J. Bartek, "The DNA-damage response in human biology and disease," *Nature*, vol. 461, no. 7267, pp. 1071–1078, Oct. 2009.

[19] N. F. Lowndes and G. W.-L. Toh, "DNA Repair: The Importance of Phosphorylating Histone H2AX," *Curr. Biol.*, vol. 15, no. 3, pp. R99–R102, Feb. 2005.

[20] H. C. Reinhardt and B. Schumacher, "The p53 network: cellular and systemic DNA damage responses in aging and cancer," *Trends Genet.*, vol. 28, no. 3, pp. 128–136, Mar. 2012.

[21] M. Podhorecka, A. Skladanowski, and P. Bozko, “H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy,” *J. Nucleic Acids*, vol. 2010, 2010.

[22] E. P. Rogakou, C. Boon, C. Redon, and W. M. Bonner, “Megabase Chromatin Domains Involved in DNA Double-Strand Breaks in Vivo,” *J. Cell Biol.*, vol. 146, no. 5, pp. 905–916, Sep. 1999.

[23] M.-C. Keogh, J.-A. Kim, M. Downey, J. Fillingham, D. Chowdhury, J. C. Harrison, M. Onishi, N. Datta, S. Galicia, A. Emili, J. Lieberman, X. Shen, S. Buratowski, J. E. Haber, D. Durocher, J. F. Greenblatt, and N. J. Krogan, “A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery,” *Nature*, vol. 439, no. 7075, pp. 497–501, Jan. 2006.

[24] D. Chowdhury, M.-C. Keogh, H. Ishii, C. L. Peterson, S. Buratowski, and J. Lieberman, “γ-H2AX Dephosphorylation by Protein Phosphatase 2A Facilitates DNA Double-Strand Break Repair,” *Mol. Cell*, vol. 20, no. 5, pp. 801–809, Dec. 2005.

[25] A. Torgovnick and B. Schumacher, “DNA repair mechanisms in cancer development and therapy,” *Front. Genet.*, vol. 6, Apr. 2015.

[26] F. Dietlein, L. Thelen, and H. C. Reinhardt, “Cancer-specific defects in DNA repair pathways as targets for personalized therapeutic approaches,” *Trends Genet.*, vol. 30, no. 8, pp. 326–339, Aug. 2014.

[27] T. Helleday, E. Petermann, C. Lundin, B. Hodgson, and R. A. Sharma, “DNA repair pathways as targets for cancer therapy,” *Nat. Rev. Cancer*, vol. 8, no. 3, pp. 193–204, Mar. 2008.

[28] H. Harashima, N. Dissmeyer, and A. Schnittger, “Cell cycle control across the eukaryotic kingdom,” *Trends Cell Biol.*, vol. 23, no. 7, pp. 345–356, Jul. 2013.

[29] D. O. Morgan, *The Cell Cycle: Principles of Control*. New Science Press, 2007.

[30] C. Bertoli, J. M. Skotheim, and R. A. M. de Bruin, “Control of cell cycle transcription during G1 and S phases,” *Nat. Rev. Mol. Cell Biol.*, vol. 14, no. 8, pp. 518–528, Aug. 2013.

[31] J. Bähler, “Cell-cycle control of gene expression in budding and fission yeast,” *Annu. Rev. Genet.*, vol. 39, pp. 69–94, 2005.

[32] B. Gabrielli, K. Brooks, and S. Pavey, “Defective cell cycle checkpoints as targets for anti-cancer therapies,” *Front. Pharmacol.*, vol. 3, p. 9, 2012.

[33] M. Malumbres and M. Barbacid, “To cycle or not to cycle: a critical decision in cancer,” *Nat. Rev. Cancer*, vol. 1, no. 3, pp. 222–231, Dec. 2001.

[34] J. Massagué, “G1 cell-cycle control and cancer,” *Nature*, vol. 432, no. 7015, pp. 298–306, Nov. 2004.

[35] R. H. Medema and L. Macůrek, “Checkpoint control and cancer,” *Oncogene*, vol. 31, no. 21, pp. 2601–2613, May 2012.

[36] J. Bartek, C. Lukas, and J. Lukas, “Checking on DNA damage in S phase,” *Nat. Rev. Mol. Cell Biol.*, vol. 5, no. 10, pp. 792–804, Oct. 2004.

[37] M. B. Kastan and J. Bartek, “Cell-cycle checkpoints and cancer,” *Nature*, vol. 432, no. 7015, pp. 316–323, Nov. 2004.

[38] G. J. P. L. Kops, B. A. A. Weaver, and D. W. Cleveland, “On the road to cancer: aneuploidy and the mitotic checkpoint,” *Nat. Rev. Cancer*, vol. 5, no. 10, pp. 773–785, Oct. 2005.

[39] A. Musacchio and E. D. Salmon, “The spindle-assembly checkpoint in space and time,” *Nat. Rev. Mol. Cell Biol.*, vol. 8, no. 5, pp. 379–393, May 2007.

[40] L. H. Hartwell and M. B. Kastan, “Cell cycle control and cancer,” *Science*, vol. 266, no. 5192, pp. 1821–1828, Dec. 1994.

[41] M. Malumbres and M. Barbacid, “Mammalian cyclin-dependent kinases,” *Trends Biochem. Sci.*, vol. 30, no. 11, pp. 630–641, Nov. 2005.

[42] L. Hayflick, “The limited in vitro lifetime of human diploid cell strains,” *Exp. Cell Res.*, vol. 37, no. 3, pp. 614–636, Mar. 1965.

[43] J. Campisi and F. d’Adda di Fagagna, “Cellular senescence: when bad things happen to good cells,” *Nat. Rev. Mol. Cell Biol.*, vol. 8, no. 9, pp. 729–740, Sep. 2007.

[44] T. Kuilman, C. Michaloglou, W. J. Mooi, and D. S. Peeper, “The essence of senescence,” *Genes Dev.*, vol. 24, no. 22, pp. 2463–2479, Nov. 2010.

[45] K. Itahana, J. Campisi, and G. P. Dimri, “Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay,” *Methods Mol. Biol. Clifton NJ*, vol. 371, pp. 21–31, 2007.

[46] G. P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, and O. Pereira-Smith, “A biomarker that identifies senescent human cells in culture and in aging skin in vivo,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 20, pp. 9363–9367, Sep. 1995.

[47] F. Debacq-Chainiaux, J. D. Erusalimsky, J. Campisi, and O. Toussaint, “Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo,” *Nat. Protoc.*, vol. 4, no. 12, pp. 1798–1806, 2009.

[48] D. J. Kurz, S. Decary, Y. Hong, and J. D. Erusalimsky, “Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells,” *J. Cell Sci.*, vol. 113 (Pt 20), pp. 3613–3622, Oct. 2000.

[49] B. Y. Lee, J. A. Han, J. S. Im, A. Morrone, K. Johung, E. C. Goodwin, W. J. Kleijer, D. DiMaio, and E. S. Hwang, “Senescence-associated beta-galactosidase is lysosomal beta-galactosidase,” *Aging Cell*, vol. 5, no. 2, pp. 187–195, Apr. 2006.

[50] A. Ventura, D. G. Kirsch, M. E. McLaughlin, D. A. Tuveson, J. Grimm, L. Lintault, J. Newman, E. E. Reczek, R. Weissleder, and T. Jacks, “Restoration of p53 function leads to tumour regression in vivo,” *Nature*, vol. 445, no. 7128, pp. 661–665, Feb. 2007.

[51] M. Collado and M. Serrano, “Senescence in tumours: evidence from mice and humans,” *Nat. Rev. Cancer*, vol. 10, no. 1, pp. 51–57, Jan. 2010.

[52] J. C. Acosta and J. Gil, “Senescence: a new weapon for cancer therapy,” *Trends Cell Biol.*, vol. 22, no. 4, pp. 211–219, Apr. 2012.

[53] M. C. Maiuri, E. Zalckvar, A. Kimchi, and G. Kroemer, “Self-eating and self-killing: crosstalk between autophagy and apoptosis,” *Nat. Rev. Mol. Cell Biol.*, vol. 8, no. 9, pp. 741–752, Sep. 2007.

[54] J. F. Kerr, A. H. Wyllie, and A. R. Currie, “Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics,” *Br. J. Cancer*, vol. 26, no. 4, pp. 239–257, Aug. 1972.

[55] A. H. Wyllie, J. F. Kerr, and A. R. Currie, “Cell death: the significance of apoptosis,” *Int. Rev. Cytol.*, vol. 68, pp. 251–306, 1980.

[56] M. Leist and M. Jäättelä, “Four deaths and a funeral: from caspases to alternative mechanisms,” *Nat. Rev. Mol. Cell Biol.*, vol. 2, no. 8, pp. 589–598, Aug. 2001.

[57] R. C. Taylor, S. P. Cullen, and S. J. Martin, “Apoptosis: controlled demolition at the cellular level,” *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 3, pp. 231–241, Mar. 2008.

[58] T. G. Cotter, “Apoptosis and cancer: the genesis of a research field,” *Nat. Rev. Cancer*, vol. 9, no. 7, pp. 501–507, Jul. 2009.

[59] D. W. Nicholson, “Caspase structure, proteolytic substrates, and function during apoptotic cell death,” *Cell Death Differ.*, vol. 6, no. 11, pp. 1028–1042, Nov. 1999.

[60] T. Shintani and D. J. Klionsky, “Autophagy in health and disease: a double-edged sword,” *Science*, vol. 306, no. 5698, pp. 990–995, Nov. 2004.

[61] P. G. Clarke, “Developmental cell death: morphological diversity and multiple mechanisms,” *Anat. Embryol. (Berl.)*, vol. 181, no. 3, pp. 195–213, 1990.

[62] J. J. Lum, D. E. Bauer, M. Kong, M. H. Harris, C. Li, T. Lindsten, and C. B. Thompson, “Growth factor regulation of autophagy and cell survival in the absence of apoptosis,” *Cell*, vol. 120, no. 2, pp. 237–248, Jan. 2005.

[63] Z. J. Yang, C. E. Chee, S. Huang, and F. A. Sinicrope, “The role of autophagy in cancer: therapeutic implications,” *Mol. Cancer Ther.*, vol. 10, no. 9, pp. 1533–1541, Sep. 2011.

[64] “He Laboratory.” [Online]. Available: http://labs.feinberg.northwestern.edu/congconghe/home/. [Accessed: 16-Apr-2016].

[65] S. Pankiv, T. H. Clausen, T. Lamark, A. Brech, J.-A. Bruun, H. Outzen, A. Øvervatn, G. Bjørkøy, and T. Johansen, “p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy,” *J. Biol. Chem.*, vol. 282, no. 33, pp. 24131–24145, Aug. 2007.

[66] R. Mathew, C. M. Karp, B. Beaudoin, N. Vuong, G. Chen, H.-Y. Chen, K. Bray, A. Reddy, G. Bhanot, C. Gelinas, R. S. Dipaola, V. Karantza-Wadsworth, and E. White, “Autophagy suppresses tumorigenesis through elimination of p62,” *Cell*, vol. 137, no. 6, pp. 1062–1075, Jun. 2009.

[67] B. Levine and D. J. Klionsky, “Development by self-digestion: molecular mechanisms and biological functions of autophagy,” *Dev. Cell*, vol. 6, no. 4, pp. 463–477, Apr. 2004.

[68] A. Takamura, M. Komatsu, T. Hara, A. Sakamoto, C. Kishi, S. Waguri, Y. Eishi, O. Hino, K. Tanaka, and N. Mizushima, “Autophagy-deficient mice develop multiple liver tumors,” *Genes Dev.*, vol. 25, no. 8, pp. 795–800, Apr. 2011.

[69] Y. Kondo, T. Kanzawa, R. Sawaya, and S. Kondo, “The role of autophagy in cancer development and response to therapy,” *Nat. Rev. Cancer*, vol. 5, no. 9, pp. 726–734, Sep. 2005.

[70] G. M. Cragg and D. J. Newman, “Antineoplastic agents from natural sources: achievements and future directions,” *Expert Opin. Investig. Drugs*, vol. 9, no. 12, pp. 2783–2797, Dec. 2000.

[71] G. M. Cragg and D. J. Newman, “Plants as a source of anti-cancer agents,” *J. Ethnopharmacol.*, vol. 100, no. 1–2, pp. 72–79, Aug. 2005.

[72] Y. Al Dhaheri, A. Eid, S. AbuQamar, S. Attoub, M. Khasawneh, G. Aiche, S. Hisaindee, and R. Iratni, “Mitotic Arrest and Apoptosis in Breast Cancer Cells Induced by Origanum majorana Extract: Upregulation of TNF-α and

Downregulation of Survivin and Mutant p53," *PLoS ONE*, vol. 8, no. 2, p. e56649, Feb. 2013.

[73] M. Lam, A. R. Carmichael, and H. R. Griffiths, "An Aqueous Extract of Fagonia cretica Induces DNA Damage, Cell Cycle Arrest and Apoptosis in Breast Cancer Cells via FOXO3a and p53 Expression," *PLOS ONE*, vol. 7, no. 6, p. e40152, Jun. 2012.

[74] J. X. Kang, J. Liu, J. Wang, C. He, and F. P. Li, "The extract of huanglian, a medicinal herb, induces cell growth arrest and apoptosis by upregulation of interferon-beta and TNF-alpha in human breast cancer cells," *Carcinogenesis*, vol. 26, no. 11, pp. 1934–1939, Nov. 2005.

[75] Y. S. Tor, L. S. Yazan, J. B. Foo, N. Armania, Y. K. Cheah, R. Abdullah, M. U. Imam, N. Ismail, and M. Ismail, "Induction of apoptosis through oxidative stress-related pathways in MCF-7, human breast cancer cells, by ethyl acetate extract of Dillenia suffruticosa," *BMC Complement. Altern. Med.*, vol. 14, p. 55, 2014.

[76] K. Manju, R. K. Jat, and G. Anju, "A review on medicinal plants used as a source of anticancer agents," *Int. J. Drug Res. Technol.*, vol. 2, no. 2, p. 177–1 83, 2012.

[77] S. Rayne and G. Mazza, "Biological activities of extracts from sumac (Rhus spp.): a review," *Plant Foods Hum. Nutr. Dordr. Neth.*, vol. 62, no. 4, pp. 165–175, Dec. 2007.

[78] J. Chrtek, "Flora of turkey and the east aegean Islands.," *P H Davis Ed Folia Geobot. Phytotaxon.*, vol. 19, no. 3, pp. 322–322.

[79] B. Abu-Shanab, G. Adwan, D. Abu-Safiya, K. Adwan, and M. Abu-Shanab, "Antibacterial activity of Rhus coriaria. L extracts growing in Palestine," *J. Islam. Stud.*, vol. 13, no. 2, pp. 147–153, Jan. 2005.

[80] I. M. Abu-Reidah, M. S. Ali-Shtayeh, R. M. Jamous, D. Arráez-Román, and A. Segura-Carretero, "HPLC-DAD-ESI-MS/MS screening of bioactive components from Rhus coriaria L. (Sumac) fruits," *Food Chem.*, vol. 166, pp. 179–191, Jan. 2015.

[81] M. Kosar, B. Bozan, F. Temelli, and K. H. C. Baser, "Antioxidant activity and phenolic composition of sumac (Rhus coriaria L.) extracts," *Food Chem.*, vol. 103, no. 3, pp. 952–959, 2007.

[82] H. Zargham and R. Zargham, "Tannin extracted from Sumac inhibits vascular smooth muscle cell migration," *McGill J. Med. MJM Int. Forum Adv. Med. Sci. Stud.*, vol. 11, no. 2, pp. 119–123, Jul. 2008.

[83] T. Anwer, M. Sharma, G. Khan, M. Iqbal, M. S. Ali, M. S. Alam, M. M. Safhi, and N. Gupta, "Rhus coriaria ameliorates insulin resistance in non-insulin-

dependent diabetes mellitus (NIDDM) rats," *Acta Pol. Pharm.*, vol. 70, no. 5, pp. 861–867, Oct. 2013.

[84] A. Shabbir, "Rhus coriaria Linn, a plant of medicinal, nutritional and industrial importance: A review," *J. Anim. Plant Sci.*, vol. 22, no. 2, pp. 505–512, 2012.

[85] A. Panico, V. Cardile, N. A. Santagati, and R. Messina, "Antioxidant and protective effects of Sumac leaves on chondrocytes," *J. Med. Plants Res.*, vol. 3, no. 11, pp. 855–861, Nov. 2009.

[86] J. Pourahmad, M. R. Eskandari, R. Shakibaei, and M. Kamalinejad, "A search for hepatoprotective activity of aqueous extract of Rhus coriaria L. against oxidative stress cytotoxicity," *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.*, vol. 48, no. 3, pp. 854–858, Mar. 2010.

[87] A. Chakraborty, F. Ferk, T. Simić, A. Brantner, M. Dusinská, M. Kundi, C. Hoelzl, A. Nersesyan, and S. Knasmüller, "DNA-protective effects of sumach (Rhus coriaria L.), a common spice: results of human and animal studies," *Mutat. Res.*, vol. 661, no. 1–2, pp. 10–17, Feb. 2009.

[88] S. Mohammadi, S. Montasser Kouhsari, and A. Monavar Feshani, "Antidiabetic properties of the ethanolic extract of Rhus coriaria fruits in rats," *Daru J. Fac. Pharm. Tehran Univ. Med. Sci.*, vol. 18, no. 4, pp. 270–275, 2010.

[89] S. M. Nasar-Abbas and A. K. Halkman, "Antimicrobial effect of water extract of sumac (Rhus coriaria L.) on the growth of some food borne bacteria including pathogens," *Int. J. Food Microbiol.*, vol. 97, no. 1, pp. 63–69, Dec. 2004.

[90] M. Mirian, M. Behrooeian, M. Ghanadian, N. Dana, and H. Sadeghi-Aliabadi, "Cytotoxicity and antiangiogenic effects of Rhus coriaria, Pistacia vera and Pistacia khinjuk oleoresin methanol extracts," *Res. Pharm. Sci.*, vol. 10, no. 3, pp. 233–240, Jun. 2015.

[91] M. A. O'Brien, R. A. Moravec, and T. L. Riss, "Poly (ADP-ribose) polymerase cleavage monitored in situ in apoptotic cells," *BioTechniques*, vol. 30, no. 4, pp. 886–891, Apr. 2001.

[92] S.-F. Zhang, X.-L. Wang, X.-Q. Yang, and N. Chen, "Autophagy-associated targeting pathways of natural products during cancer treatment," *Asian Pac. J. Cancer Prev. APJCP*, vol. 15, no. 24, pp. 10557–10563, 2014.

[93] M. Huovinen, J. Loikkanen, P. Myllynen, and K. H. Vähäkangas, "Characterization of human breast cancer cell lines for the studies on p53 in chemical carcinogenesis," *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA*, vol. 25, no. 5, pp. 1007–1017, Aug. 2011.

[94] L. Zhang, C. Hao, G. Dong, and Z. Tong, “Analysis of Clinical Features and Outcome of 356 Triple-Negative Breast Cancer Patients in China,” *Breast Care*, vol. 7, no. 1, pp. 13–17, Feb. 2012.

[95] B.-D. Chang, M. E. Swift, M. Shen, J. Fang, E. V. Broude, and I. B. Roninson, “Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 1, pp. 389–394, Jan. 2002.

[96] S. Sridharan, K. Jain, and A. Basu, “Regulation of Autophagy by Kinases,” *Cancers*, vol. 3, no. 2, pp. 2630–2654, Jun. 2011.

[97] H. Rodriguez-Rocha, A. Garcia-Garcia, M. I. Panayiotidis, and R. Franco, “DNA damage and autophagy,” *Mutat. Res.*, vol. 711, no. 1–2, pp. 158–166, Jun. 2011.

[98] U. Herbig, W. A. Jobling, B. P. C. Chen, D. J. Chen, and J. M. Sedivy, “Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a),” *Mol. Cell*, vol. 14, no. 4, pp. 501–513, May 2004.

[99] D. A. Gewirtz, “Autophagy and senescence: a partnership in search of definition,” *Autophagy*, vol. 9, no. 5, pp. 808–812, May 2013.

[100] G. Mosieniak, M. Adamowicz, O. Alster, H. Jaskowiak, A. A. Szczepankiewicz, G. M. Wilczynski, I. A. Ciechomska, and E. Sikora, “Curcumin induces permanent growth arrest of human colon cancer cells: Link between senescence and autophagy,” *Mech. Ageing Dev.*, vol. 133, no. 6, pp. 444–455, Jun. 2012.

[101] S. Patschan, J. Chen, A. Polotskaia, N. Mendelev, J. Cheng, D. Patschan, and M. S. Goligorsky, “Lipid mediators of autophagy in stress-induced premature senescence of endothelial cells,” *Am. J. Physiol. Heart Circ. Physiol.*, vol. 294, no. 3, pp. H1119-1129, Mar. 2008.

List of Publications

H. El Hasasna, K. Athamneh, H. Al Samri, N. Karuvantevida, Y. Al Dhaheri, S. Hisaindee, G. Ramadan, N. A. Tamimi, S. AbuQamar, A. Eid, and R. Iratni, “Rhus coriaria induces senescence and autophagic cell death in breast cancer cells through a mechanism involving p38 and ERK1/2 activation,” *Sci. Rep.*, vol. 5, p. 13013, Aug. 2015.

H. El Hasasna, A. Saleh, H. Al Samri, K. Athamneh, S. Attoub, K. Arafat, N. Benhalilou, S. Alyan, J. Viallet, Y. Al Dhaheri, A. Eid, and R. Iratni, “Rhus coriaria suppresses angiogenesis, metastasis and tumor growth of breast cancer through inhibition of STAT3, NFκB and nitric oxide pathways,” *Sci. Rep.*, vol. 6, p. 21144, Feb. 2016.